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## Synthetic Organic Local Anesthetics*,†

*By Marjorie B. Moore*

The literature on local anesthetics might well be described as voluminous. Hence, a reasonably brief review must be written from a definite point of view and with certain objectives in mind. In the present instance the point of view is that of a pharmaceutical chemist, and the attempt is to discuss compounds with local anesthetic action as related to their chemical structure. In some cases the practical value of such compounds is discussed, but compounds are included irrespective of this criterion, provided only that they are capable of exerting local anesthetic effect. No claim is made that all such compounds are included, but mention of all which are of particular theoretical or practical interest has been attempted. The literature cited is in the English language wherever possible, U. S. patents being cited in preference to their foreign counterparts. Earlier references are often omitted if they are summarized in more recent publications. The patent literature has been used frequently because the patenting of a compound is evidence that it was considered to be of possible practical value.

A number of reviews on the general subject of local anesthetics have been published, usually from the point of view of the pharmacologist or clinician (1, 2, 3, 4, 5, 6); those by Miescher (7) and Schaumann (8) from the chemical point of view as well. These reviews should be consulted for methods of pharmacological testing, and for clinical requirements of local anesthetics for various purposes.

Beutner and Calesnick (4) state, "The conclusion may be drawn, therefore, that *no* compound without an ester structure can be an efficient nonirritant local anesthetic agent." Without necessarily disagreeing with this conclusion (although the authors appear to consider amides as esters), it still may be noted that a wide variety of structures are capable of exerting local anesthetic effect. This is in marked contrast to many physiologic effects, for which a single or a very limited group of compounds is specific. The lack of specificity for local anesthetic action may well be illustrated by the compounds listed in "New and Nonofficial Remedies," 1943 edition: twelve are esters, one an amide, one a phenylurethane, one an amidine and one an alcohol. Of the esters, one is an ester of cinnamic acid, two of benzoic acid, eight of *p*-aminobenzoic acid, and one of *p*-butylaminobenzoic acid; seven are dialkylaminoalkyl esters, one a bis-(dialkylamino)-alkyl ester, one a monoalkylaminoalkyl ester, and three alkyl esters.

*Alkyl Esters of Aromatic Acids.*—Alkyl esters of aminobenzoic acids, represented in the above list by ethyl and butyl *p*-aminobenzoates (Anesthesin and Butesin)

* Received April 28, 1944, from Abbott Laboratories, North Chicago, Ill.

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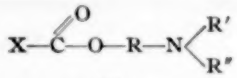
and by methyl *p*-amino-*m*-hydroxybenzoate (Orthoform-new), are very sparingly soluble in water and hence are most useful for local application, particularly to denuded surfaces. Butesin Picrate is recommended especially for the treatment of burns.

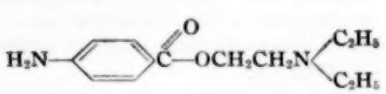
*Alkamine Esters of Aromatic Acids.*—By far the most numerous list of compounds synthesized for local anesthetic purposes are the alkamine esters of aromatic acids. They have furnished the preponderant number of marketable local anesthetics. They may be represented by the general formula



(I)

in which X is an aromatic ring, substituted or unsubstituted; R is a divalent alkylene radical; R′ and R″ are hydrocarbon radicals or hydrogen, or together may represent the remaining parts of a heterocyclic aliphatic ring of which the nitrogen is a member and which may contain a second heteroatom. The advantage of this group lies in the ability of the aliphatic amino nitrogen to form salts with simple inorganic acids, thus conferring water solubility. These compounds thus become suitable for injection and other uses for which such solubility is necessary. In discussion of local anesthetic compounds containing an amino group, the formulas will be written without including that of the acid used to form a salt. In nearly all cases, and unless specifically mentioned, it is understood that the hydrochlorides of the bases have been prepared for pharmacologic and clinical use. Various workers have claimed variations in anesthetic activity or stability due to the acid combined with a certain base (11, 12, 13), but no such salts have come into common use except Procaine Borate, N. N. R., and the advantages of this over Procaine Hydrochloride are open to question (14). Procaine Nitrate, N. N. R., is used for its compatibility with silver salts, and Butyn is sold as the sulfate because of the relative insolubility of the hydrochloride.

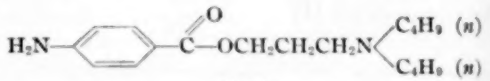
The classic examples of the type represented by Formula I are due to the work of Einhorn (9, 10), the inventor of Procaine (Novocain),



(II)

which was introduced in 1905, and even yet is more widely used for injection than any other anesthetic. The compounds which he synthesized included dialkylaminoalkyl esters of *o*-, *m*- and *p*-aminobenzoic acids, as well as bis-(dialkylamino)-alkyl esters.

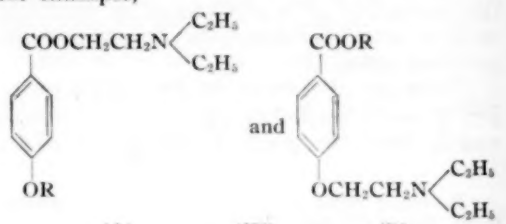
While Procaine is relatively low in toxicity and quite effective when given hypodermically, it requires the addition of a vasopressor substance for consistent results, and it is only very slightly active as a topical anesthetic. Hence, many compounds of the same general type have been synthesized in the effort to improve these properties. Some have been successful, especially in conferring topical anesthetic activity. Butyn (Butacaine, U. S. P.)



(III)

is a very effective topical anesthetic.

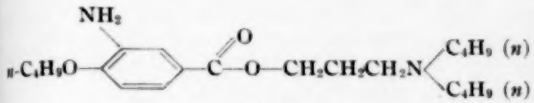
In the search for improved local anesthetics, many modifications of Formula I have been tried. The acid portion of the molecule has been varied in several ways, for instance, in the kind and position of substituents on the benzene ring. Thus, in addition to simple benzoates and aminobenzoates, esters of hydroxybenzoates (15, 16, 17, 18, 19), alkoxybenzoates (16, 17, 19, 20, 21, 22, 23), alkylmercaptobenzoates (24, 25, 26), aryloxybenzoates (27) and aminoalkylbenzoates (28) have been studied. There may, of course, be more than one such substituent, or a combination, as of hydroxy and alkyl (18) or of amino and alkoxy (29). In place of simple aminobenzoates, the nitrogen on the aromatic amino group may be substituted by alkyl (30, 31), alkoxyalkyl (31), hydroxyalkyl (32), dialkylaminoalkyl (33) or dialkylaminoacyl (34) groups. Other substituents studied have been halogens with (35, 36, 37) or without (23, 38, 39) the simultaneous presence of an amino group on the ring. Rohmann and Koch (40) compared isomeric compounds which were dialkylaminoalkyl esters of alkoxybenzoic acid and alkyl esters of dialkylaminoalkoxybenzoic acid, for example,



(A) (IV) (B)

Series (A) compounds of the same molecular weight were found to be effective in lower

concentration than those of Series (B), but all are quite efficient local anesthetics. There is no doubt that an alkyl group in an alkoxy substituent on the ring exerts a profound effect upon the local anesthetic properties. Larger alkyl groups, especially in conjunction with an amino group as in



(V)

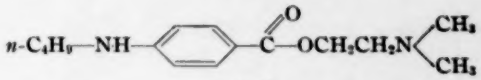
give extremely powerful local anesthetics (41). Likewise, an alkyl group on the aromatic amino nitrogen exerts a profound effect upon the anesthetic potency, as in Tetracaine, U. S. P. (Pontocaine), (Formula VI), which is a powerful anesthetic in spite of the small ester chain. Unfortunately, in both cases, the toxicity is greatly increased over less active types.

In place of the benzene ring, other isocyclic rings have been substituted, as naphthalene (42, 43, 44, 45, 46), biphenyl (47, 48, 49) or anthracene (50). As in the benzene series, the substituents in these other aromatic rings may be varied, usually with similar general effect upon local anesthetic action.

Heterocyclic aromatic rings have also been studied as X in Formula I, esters of carboxylic acids of pyridine (51, 52, 53), pyrrole (54, 55), furan (55, 56, 57, 58), thiophene (55, 58, 123), thiazole (58), carbazole (59, 60), dibenzofuran (59), dibenzothiophene (59) and quinoline (61, 62, 63). In the case of heterocyclic rings, the position of the carboxyl relative to the hetero-atom and to other substituents may have a marked effect upon anesthetic activity. Alkamine esters of unsubstituted pyridine carboxylates are very low in activity (51, 52), but this may be increased by such substituents as amino or alkoxy (53). Whereas Lurje (62) found esters of 2-alkoxyquinoline-4-carboxylic acid to be strong anesthetics, Moness and Christiansen (63) report that an ester of 8-alkoxyquinoline-5-carboxylic acid was only slightly anesthetic. The effect of the hetero-atom in the ring varies, but in general heterocyclic rings do not give as active derivatives as do isocyclic rings. Gilman and Pickens (55) give the order of decreasing activity as shown in Formula VII.

The order given by Dann (58) for the thiophene and furan analogues of Procaine agrees with this; thiophene and furan analogues of Anesthesin were approximately equal, while the thiazole analogue was inactive. Cook and Kreke (57) found esters of 2-furoic acid and of benzoic acid to be approximately equal in anesthetic activity, with the furoates more active in some cases.

A great variety of aminoalcohols has been used to form esters with aromatic acids, giving greater or less anesthetic activity. The most common types are those in which R′ and R″ in Formula I are the same, usually lower alkyl groups. Examples of this type from the list of local anesthetics in "New and Nonofficial Remedies" are: Procaine, Butacaine, Larocaine, Tetracaine and Tutocaine. The references already cited abound in examples of this type and will not be separately listed here.

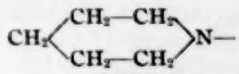


(VI)

Benzene Pyrrole Thiophene Furan

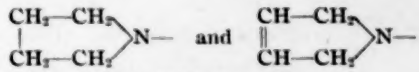
(VII)

If R′ and R″ (Formula I) together with the nitrogen form an aliphatic heterocyclic ring, many variations are possible. The piperidine ring,



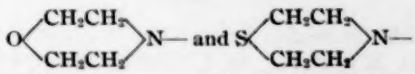
(VIII)

is perhaps the most obvious, and many compounds have been prepared, including piperidine or substituted piperidines (64, 65, 66, 67, 68). Metycaine, N. N. R., is an example of this type. Other aliphatic rings which have been studied in this connection (54, 70) are



Pyrrolidine (IX) Pyrroline

and, with a second hetero-atom (69, 70),

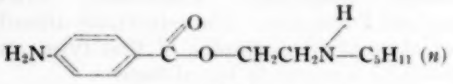


Morpholine (X) Thiomorpholine

Compounds in which R′ and R″ are identical and unsaturated have been patented (71). Those in which R′ and R″ are different and saturated were studied by Brill (72); in which they are different and one

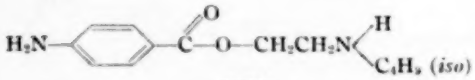
contains an ethylenic linkage by von Braun and Braunsdorf (73) and by Volwiler and Adams (74); and those in which they are different and one is hydroxyalkyl have recently been patented (75).

Esters in which either R′ or R″ of Formula I is H have been neglected for some time, but have recently received much study (76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86). Amylcaine, N. N. R., is a representative of this type, useful principally as a topical anesthetic,



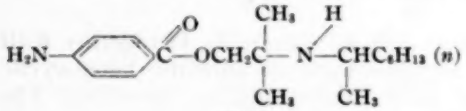
(XI)

Monocaine,



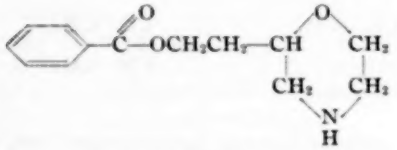
(XII)

has been recommended for injection anesthesia in dentistry, and its formate has been used for spinal anesthesia (85). Octacaine,



(XIII)

has been suggested as a promising surface anesthetic and a possibly valuable agent for tissue and spinal anesthesia (86). Included in this group in which either R′ or R″ is H must be those compounds in which the other is cyclic and aliphatic as in piperidine derivatives (87, 88, 89), morpholine derivatives (90, 91) or reduced quinoline derivatives (92) not connected through the nitrogen and thus leaving a secondary amine in the ring, as



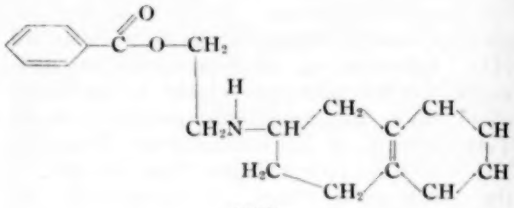
(XIV)

To this class also belong the compounds in which R′ or R″ is a tetrahydronaphthyl group (93, 94), as shown in Formula XV.

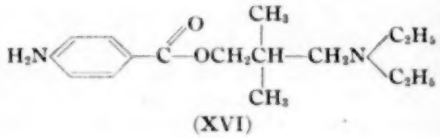
Little study has been made of the case in which both R′ and R″ are H, *i. e.*, a primary amino group on the alkylene. However, Forster and Fierz (95) reported that β-aminoethyl-*p*-aminobenzoate showed no anesthetic effect in five per cent solution.

R, the divalent alkylene radical of Formula I, may be either straight or branched (96). The former is represented in "New and Non-official Remedies" by such compounds as Procaine and Butyn, and the latter by Larocaine (Formula XVI).

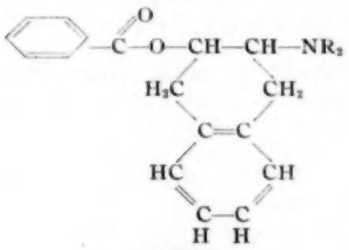
For the case in which R is $-CH_2-$, the compounds are effective, but usually irritating (97). R may also contain a cyclic structure, as in esters of cyclohexanes (98) or tetrahydronaphthalenes (99) with alkamine side chains (Formula XVII).



(XV)

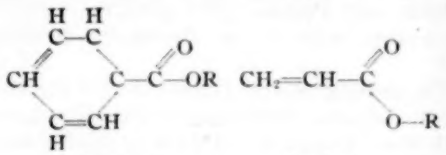


(XVI)



(XVII)

*Esters of Aliphatic Acids.*—Esters other than those conforming to the above type formula have been prepared. Thus it was shown that alkamine esters of simple saturated lower aliphatic acids exert no local anesthetic effect (100). However, Brill and Bulow (101) showed that diethylaminoethyl esters of some higher saturated acids exerted a narcotic effect upon goldfish. Alkamine esters of acrylic and other unsaturated acids having a double bond on the same carbon as the carboxy do show a definite anesthetic effect. The structural formulas show the relationship to benzoates.

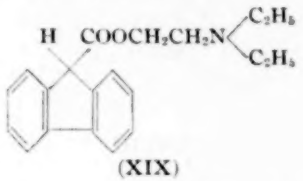


Benzoate Acrylate

(XVIII)

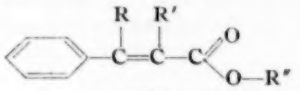
However, β-aminoacrylates are not local anesthetics (103). Alkamine esters of aliphatic acids with a triple bond in the same position likewise are anesthetic (102). None of the purely aliphatic esters has shown sufficient activity to be of any practical value.

*Esters of Aromatic Aliphatic Acids.*—While purely aliphatic acids are of little interest for the formation of esters with anesthetic properties, the introduction of an aryl group not directly connected to the carboxyl may give compounds of greater value. Esters of aminomandelic acids have only weakly anesthetic properties (104, 105), as do those of phenylacetic and related acids, whose alkamine esters are principally useful for their antispasmodic action (106). Lehmann and Knoefel (50) tested cyclic compounds belonging to this type, as esters of 9-fluorene carboxylic acid,



(XIX)

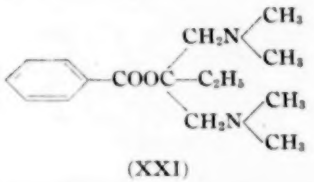
and found them to be fairly effective local anesthetics. If the acid contains a double bond conjugated with the aromatic ring, its esters may be powerful local anesthetics. The most striking examples are the cinnamates, or β-phenylacrylates (107, 108, 109, 110, 111),



(XX)

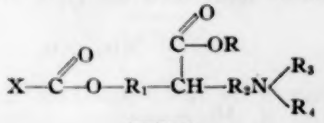
in which R and R′ may be H or alkyl and R″ is an alkamine. If a triple bond is substituted for the double bond, the corresponding esters are almost inactive, as are the analogous saturated compounds (107).

*Esters of Varied Aminoalcohols.*—Changes in the aminoalcohols used for esterification which depart from the general Formula I have been numerous, and some have given valuable local anesthetics. For instance, "New and Nonofficial Remedies" contains an example of a bis-(dialkylamino)-alkyl ester, Alypin,



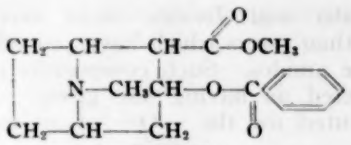
(XXI)

This and other related compounds have been covered by patents (112, 113). Instead of an alkylene R, the chain may include another atom, as oxygen (114, 115, 116, 117), or a second ester group may be present, usually an alkyl ester, giving compounds of the type,



(XXII)

where X is an aromatic ring, $R_1$ and $R_2$ are alkylene radicals and R, $R_3$, $R_4$ are alkyl groups, of which $R_3$ and $R_4$ together may represent the remainder of a heterocyclic ring of which the N is a part (118, 119, 120, 121). Cocaine,



(XXIII)

may be considered to belong to this type, as well as its furan or thiophene isologs (122, 123, 124). In this group the furoate is least active (124).

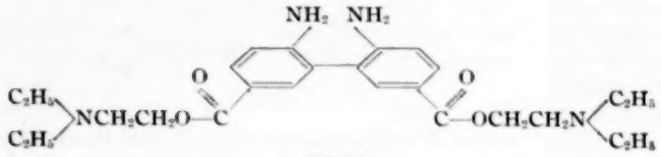
The type of Formula I may likewise be varied by introduction of an aromatic ring at some position in R (93, 125, 126, 127, 128, 129, 130, 131, 132, 133). Many of these were prepared in the hope of combining local anesthetic and sympathomimetic actions (93, 125, 128, 129, 131), but in general one or the other effect is suppressed so that the compounds are not sufficiently active to be useful for both purposes.

An aryl or an aralkyl group has been introduced in the positions R′ or R″ of Formula I (134, 135, 136, 137, 138).

Instead of simple aminoalcohols, compounds containing two or more hydroxyl groups may be esterified with the same (139, 140, 141, 143), or different (142) acids. Or dicarboxylic acids may be used to form esters with the same (144, 145, 146, 147, 148) or different (149, 150) alcohols. The compound (Formula XXIV) is said to be nonirritating and to give longer anesthesia without epinephrine than does procaine with epinephrine (146). It is, however, more toxic than procaine.

*Esters of Thiocarboxylic Acids.*—Aside from the compounds which are esters of aromatic carboxylic acids, there are a number of structures which may exert powerful local anesthetic action. One of the simplest variations is the substitution of sulfur for oxy-

gen, giving thiolbenzoates (Formula XXV). These have been studied by several groups (151, 152, 153, 154, 155). Their local anesthetic action and toxicity are not very different from those of their oxygen analogues; however, their tendency to cause dermatoses will probably limit their use (154, 156).



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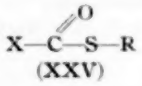
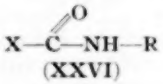


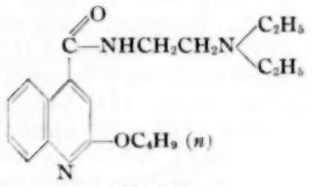
(XXV)

*Amides and Imides.*—Acid derivatives other than esters which have proved useful are the amides. Such compounds may be considered as having the group —NH— substituted for the —O— of an ester, as



(XXVI)

Among the benzamides, the most powerful appear to be those in which R is a dialkylaminoalkyl group (157). Alkyl amides of *p*-aminobenzoic acid in which the alkyl contains 4 to 8 carbons are reported to be strong surface anesthetics (158). *N*-Monomethyl amides of fatty acids containing 7, 8 or 9 carbon atoms have local anesthetic properties (159). *N*-Alkamine substituted amides of cinnamic or alkyl substituted cinnamic acids have been patented (160), and alkamine substituted amides of aminonaphthalene carboxylic acids exhibit local anesthetic activity (161). Nupercaine, N. N. R.,



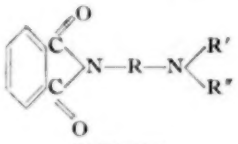
(XXVII)

is an example of an *N*-alkamine substituted amide of a quinoline carboxylic acid. Again in this series the butyl ether group on the ring gives a very powerful anesthetic compound, and also a highly toxic one.

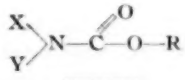
*N*-Alkamine substituted imides of dicarboxylic acids, as *N*-alkamine substituted phthalimides, are effective local anesthetics (162), as shown below in Formula XXVIII, in which R is an alkylene or substituted alkylene and R′ and R″ are alkyls.

*Urethanes.*—Other derivatives related to acids which give useful local anesthetics are the carbamates or urethanes, as shown in Formula XXIX.

Nearly all possess local anesthetic properties in greater or less degree. Many of the compounds studied are those in which X or Y is an aryl group of the benzene series. Fromherz (163) made a study of this type of compound and more recent reports have been published (164, 165, 166, 167). Others have made X or Y an aralkyl group (168, 169) or a dialkyl-

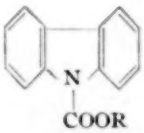


(XXVIII)



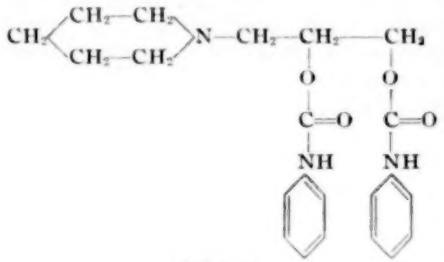
(XXIX)

aminoalkyl group (170). Or X may be H, and Y an 8-quinoline group (171). Both X and Y may be aromatic (172, 173) or they may be a part of the ring together with the nitrogen, as in carbazole *N*-carboxylates (174).



(XXX)

Another type extensively studied consists of the di-(phenylurethanes) of amino dihydroxyalcohols (175, 176). To this class belongs Diothane, N. N. R.,

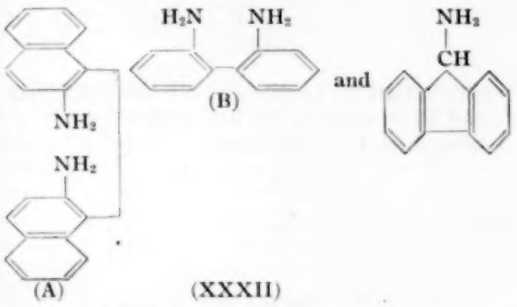


(XXXI)

Instead of esters of carbamic acids, their amides may be used (177, 178).

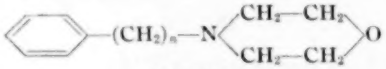
*Compounds Not Derived from Carboxylic Acids.*—Esters of sulfanilic acid have been reported to possess anesthetic properties (179).

An organic compound need not be a derivative of an acid in order to possess local anesthetic properties. It is not even necessary that nitrogen be a part of the molecule. Simple aralkyl alcohols, as benzyl alcohol, N. N. R., may have value as local anesthetics. Tetrahydrofuran has been reported as anesthetic (180). By far the greater number of anesthetic compounds, however, contain one or more nitrogen atoms. As mentioned before the aliphatic amino groups are particularly valuable for their ability to form salts and thus give greater solubility. Such compounds may have no other functional group than the amino (181, 182, 183) as in




(XXXII)

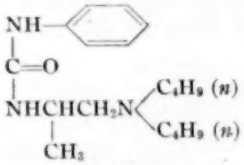
(A) is reported to be about thirty times as active as cocaine (181) and (B) about a fourth as active (183). A single amino group may be combined with another functional group, as an alcoholic hydroxyl (184, 185, 186, 187) or a carbonyl (188, 189, 190, 191) or an ether linkage (192, 193). In these cases the structure which exerts local anesthetic effect may be quite specific, as in the series reported by Leffler and Volwiler (193),




(XXXIII)

in which if $n = 1$, the compound is anesthetic, but not when $n = 2$ or 3.

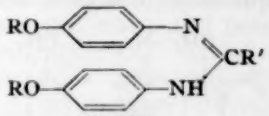
Proceeding to compounds with more than one nitrogen atom, there are the substituted ureas (194). The hydrochloride of




(XXXIV)

was found anesthetic on the tongue.

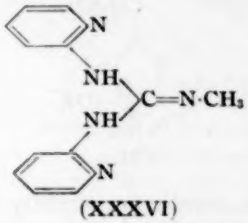
The anesthetic properties of amidines are better known. Phenacaine (Holocaine), N. N. R., is a substituted amidine of the type




(XXXV)

in which R is ethyl and R′ is methyl. A similar compound in which R is allyl has been patented (195). Variations in R and R′ have been studied (196, 197), including those in which R′ is carbalkoxyalkyl (197) or in which the R's are different and may be carbalkoxy (198).

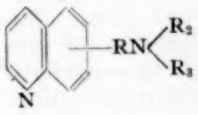
In view of the activity of amidines, it is not surprising that substituted guanidines should likewise possess local anesthetic properties. Dipyridylmethylguanidine (Protocaine)

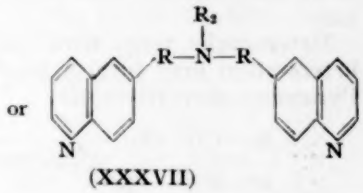



(XXXVI)

and related compounds have been found to be fairly effective local anesthetics (199, 200, 201).

Heterocyclic rings with various substituents in the proper positions may be powerful local anesthetics. Such simple compounds as α-aminopyridine or its acyl derivatives may have marked local anesthetic action (202, 203, 204). The quinoline series has been extensively studied by Kermack, *et al.* (205, 206), and by Sinha (207). Their compounds were mostly of the types

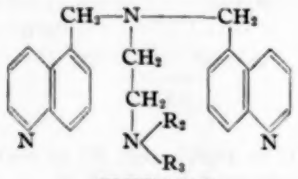



(XXXVII)

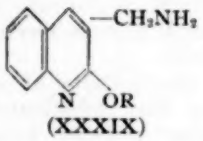
where R is a divalent radical, alkylene or alkyleneaminoalkylene, $R_2$ and $R_3$ are alkyls or may form part of a heterocyclic ring of which nitrogen is a member. Of the

series (XXXVIII), the types of compounds were very active anesthetics. The effect of ether substituents upon alkamine substituted quinolines has been studied (208).



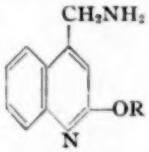
(XXXVIII)

In this series,



(XXXIX)

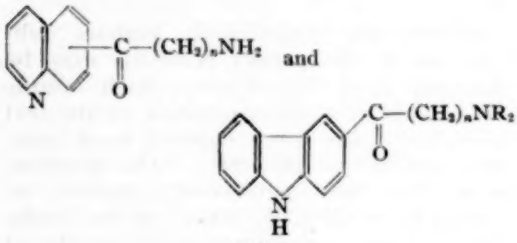
are not anesthetic, whereas



(XL)

are strongly anesthetic, slightly more than Nupercaine when R is butyl.

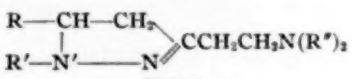
Aminoketones of quinoline and of carbazole,



(XLI)

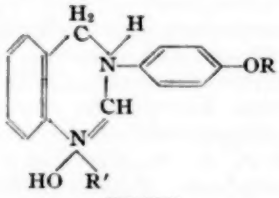
have been prepared, the carbazole derivatives being potent anesthetics (209). Eucupin, which is especially valuable for prolonged anesthesia (210), may be considered as an alkamine and ether substituted quinoline.

Heterocyclic rings with more than one hetero-atom may furnish local anesthetics. Pyrazoline derivatives, as



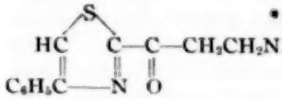
(XLII)

in which R and R′ are aryl and may be ether substituted and R″ is alkyl, are effective local anesthetics (211, 212, 213). The dihydroquinazoline ring may likewise form the basis for anesthetic compounds (214)

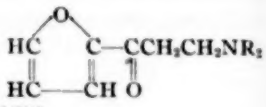


(XLIII)

Compounds of the types

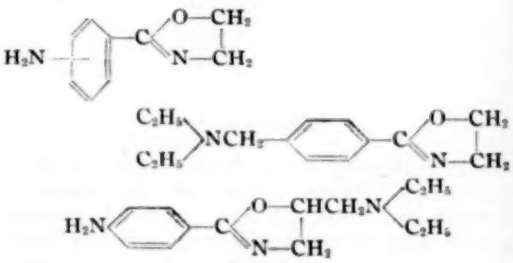


and



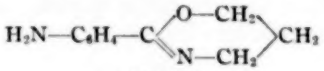
(XLIV)

were studied by Levvy and Nisbet (212) and found to be anesthetic, but irritating. Aminophenyl-2-oxazolines,



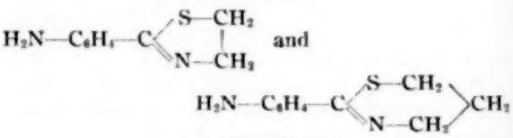
(XLV)

all show local anesthetic properties (215, 216). Aminophenyl-2-pentoxazolines (217)



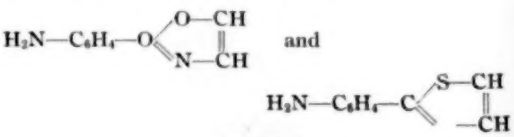
(XLVI)

thiazolines and thiazines (218),



(XLVII)

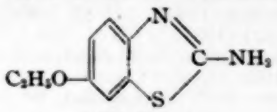
oxazoles and thiazoles (219),



(XLVIII)

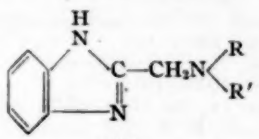
likewise are local anesthetics. A pharma-

cological study of the following compound



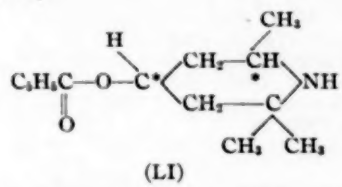
(XLIX)

showed it to be a powerful local anesthetic (220). A number of patents have been issued for local anesthetics derived from imidazoles, thiazoles, thiazolines and oxazolines (221), and their pharmacologic properties have been reported (222). The pharmacology of benzimidazoles was reviewed by Bloom and Day (223), and the preparation of 2-alkyl-aminobenzimidazoles described,



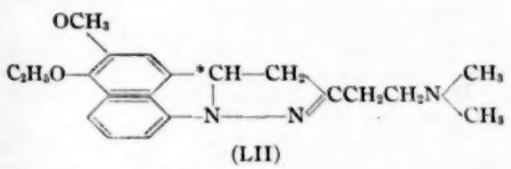
(L)

*Stereoisomerism.*—Cocaine, a natural product, occurs in an optically active form, and it might be anticipated that local anesthetics containing an asymmetric carbon atom would show a much greater activity in one or the other diastereoisomer. Here again, however, is emphasized the nonspecificity of local anesthetic effect, for in the few cases studied, the *d*-, *l*- and *dl*-forms all appear to be equally anesthetic although there is evidence for differences in toxicity. Such conclusions are based upon a limited number of cases, but of varying types. β-Eucaines and iso-β-eucaines were studied by King (224).



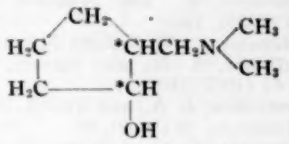
(LI)

Two racemic and four optically active forms were prepared. There was some difference in anesthetic activity between the two racemic forms, but no differences between the optical antipodes of each could be observed. Sinha (213) could show no definite differences in activity between the optical isomers of



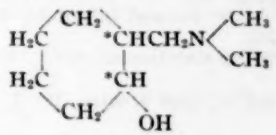
(LII)

Raasch and Brode (225) prepared *d*- and *l*- forms of Diothane and found no marked differences in local anesthetic activity between the two isomers. Schultz and Barbour (226) studied the optical isomers of cinnamylephredrine, which is a potent, but irritating, local anesthetic. Again, the local anesthetic properties of the optically active forms showed no distinct difference, although the *l*- form was only one-half as toxic for mice as was the *d*- form. In the work of King (224) it was noted that, while optical isomerism had little or no effect upon local anesthetic action, there was a difference between the two racemic forms. That stereoisomerism other than optical isomerism may affect the anesthetic properties is confirmed by the work of Mannich and Schaller (227) upon the two racemic forms of



(LIII)

In this case the benzoate of one form is very active and that of the other almost inactive. The same difference had been found by Mannich (228) for the benzoates of the two racemic forms of



(LIV)

From all the tremendous volume of experimental work on local anesthetics, it is impossible to draw any definite conclusion except that previously expressed: that a great variety of structures are capable of exerting such action. While most of these have no practical usefulness because of toxicity; irritation, low water solubility or other disadvantages, the possibility remains that some new group of compounds may yet be discovered which will more nearly approach the "ideal" anesthetic than any available at present.

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# The Origin and Function of the Alkaloids in *Mahonia nepalensis* DC. (*Berberis nepalensis Spreng.*)*

*By R. Chatterjee*

It is well known that alkaloids occur in several plant families and many investigations have been made to determine their biological significance in plants. Although researches in this line have been sporadic and the results sometimes contradictory, yet the alkaloids have come to be widely regarded as waste products of metabolism, especially for the more complex members of the group of alkaloids. The simple ones, it has been found in some cases, can be utilized by the plants which form them (1); *e. g.*, nicotine is utilized by the tobacco plants as a source of nitrogen (2). Chaze (3) finds that in the seedling nicotine is present in the vacuoles of all active cells, but that in mature plants it tends to migrate to the peripheral parts as a product of protein breakdown. The formation of alkaloids from protein breakdown products is supported by the observation of Weevers (4) that in etiolated *Ricinus* seedlings, where considerable protein breakdown occurs, the ricinine content increases, and by Sukhorukhov and Borodulina (5) working with *Datura stramonium* and *D. meteloides*. The observation of Cromwell (6), who showed by ringing experiments on *Berberis Darwinii* grown in light that berberine arises indirectly from protein breakdown products, has been supported by the present author working on the same line with *Mahonia nepalensis*, where the alkaloids, umbellatine and neprotine (7), are found to accumulate in greater amount in the stems above the ring compared with the amount of alkaloids in these tissues in normal plants, indicating that synthesis of alkaloids has taken place from products that are normally translocated from the leaves down to the root system.

Although the conclusion based on the observations of Weevers (4), and of Sukhorukhov and Borodulina (5), previously mentioned, and those of Sabalitschka and Jungermann (8) that the alkaloidal content of the germinating seed of *Strychnos nux vomica* depends on the reserve protein of the seed as a source of nitrogen, seem to establish well that the alkaloids are formed as a result of the degradation of protein, yet Henry (9) states "that the technique of such experiments is not easy and that the results seem sometimes to be susceptible of more than one interpretation." The widely accepted generalization that the alkaloids are waste products of plant metabolism was first put forward by Pictet (10) who considered that alkaloids in plants were waste products analogous to urea, ethereal sulfates, etc., of animal metabolism, and he assumed that they are produced by the decomposition of proteins into simpler basic substances and subsequent condensation of these bases with aldehydes, the active products of metabolism forming the alkaloid nucleus. The idea of the formation of the alkaloids from the products of protein breakdown has also been stressed by Robinson (11), who took up the generally accepted view that amino acids and carbohydrates (12) are the most probable starting materials for the majority of phytochemical syntheses. The view of Robinson has received support from the extensive study of Weevers (4), who found that the alkaloids which are well known chemically could all be formed in more or less complicated ways, by biochemically possible reactions from the produts of protein breakdown. Späth and Berger (13) and Hahn and Schales (14) have successfully attempted to copy nature's way of constructing tetrahydro-isoquinoline ring system, the skeleton present in umbellatine, as is found by the author of this paper (15).

An attempt has been made in this investigation to unify some of the earlier findings on the factors influencing alkaloidal content of plants. Thus Mothes (16) showed that the concentration of nicotine increased with the development of the leaves of *Nicotiana*. Koenig (17) showed that the production of alkaloids increased when nitrogen fertilizers were applied, while Annett (18) found that nitrogenous manure increased the yield of latex but not the morphine content in the opium poppy (*Papaver somniferum*). During the period of active growth of the plant, morphine was produced at the same rate as the plant tissue. The plant no longer produced morphine, when the seed began to ripen; *i. e.*, when the period of active growth ceased, and at the same time the morphine content did not diminish. It has been found that in *M. nepalensis*, the alkaloids are formed during the period of active growth and are accumulated in the cortex and bark. It appeared to Annett that morphine in the opium poppy was a useless excretory end product of metabolism, since the amount of

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morphine was dependent upon the amount of plant growth. It has been determined that the alkaloids in *M. nepalensis* are not broken down in the winter so that the total alkaloidal content increases from year to year. Like Ripert (19), who studied the effect of etiolation on the atropine and hyoscyamine content of young shoots of *Atropa*, considerable increase of alkaloids in etiolated shoots of *M. nepalensis* has been found.

The experiments, recorded in the present paper, have been planned to throw light on (*a*) the variation in the alkaloidal content of the plants with age and season, (*b*) the influence of feeding the plants with different fertilizers, (*c*) the effect of starvation, (*d*) the changes brought about by etiolation and (*e*) by ringing of the bark.

It will be seen in the present work that the results obtained lend support to Pictet's (10) generalization that alkaloids are waste products of plant metabolism. The results also establish in some cases an indubitable connection between protein breakdown and the production of alkaloids.

## EXPERIMENTAL

*The Materials.*—The plants were reared in the college and the private garden of the author and their ages were calculated from the time of the first transplantation. The root, stem and bark of the plants were first sun dried for a week or so, and then cut into small pieces and dried at 100–105° C., for twelve hours, finely powdered and dried for another twelve hours.

*Estimation of the Total Alkaloid.*—The powdered and well-dried material (10 Gm.) was extracted by means of a Soxhlet extractor with alcohol, until the extract was no longer yellow in color. The alcoholic extract was evaporated to dryness on a water bath and the residue was warmed with water (100 cc.) and filtered from resinous matter. The brown-colored aqueous solution was cooled and then extracted twice with petroleum ether (b. p. 40–60° C.), followed by three extractions with ether. The aqueous solution which was now clear and transparent was evaporated on a water bath to a small bulk (50 cc.), filtered and then treated with hydriodic acid solution until the precipitation was complete. The precipitate of the hydriodide of the alkaloids, which was golden yellow in color with a few grains of red-colored hydriodide (of the red base, neprotine), was allowed to stand overnight and filtered into a previously weighed glass Gooch crucible, washed well with water, and dried at 100–105° C. and weighed.

### A. THE VARIATION OF THE ALKALOID CONTENT WITH AGE AND SEASON

The alkaloid contents of different tissues, whole root, bark and stem wood, of both two- and four-year-old plants, were estimated periodically throughout a year. The results, shown in Table I, were obtained in six plants, supposed to be two or four years old.

The distribution of alkaloids in different tissues and the seasonal variation of the alkaloidal content of a mature plant, which had flowered and borne fruits and whose age was believed to have been about fourteen years, were also estimated.

Table I shows the increase in alkaloidal content with increase in age. The highest concentration of the alkaloids occurs in the roots of plants, young and old, and the stem-wood contains the least quantity.

*Interpretation of the Results.*—The seasonal variation of the alkaloids, as recorded in Table I, shows that the alkaloidal content increases after the period of active growth during August to October; following that period the accumulation of alkaloids is highest during winter months, when the fruits are already ripened. With the coming of the spring during March, new shoots sprout and the alkaloidal content gradually decreases till the active growth of the organs of plants is complete after the rains, during September and October. Then the alkaloidal content rises slowly until the spring comes, when a fresh growth cycle begins. These findings seem to show a connection between the utilization of protein in the growth of the plants and the synthesis and storage of alkaloids in the plants. The increase in amount of alkaloids with age, points in all probability to the fact that the alkaloids in this case are waste products of the plant metabolism.

Alkaloids were detected in old leaflets; the quantity was not great enough to be estimated gravimetrically.

### HISTOLOGICAL DISTRIBUTION OF ALKALOIDS IN VARIOUS TISSUES OF BOTH YOUNG AND OLD PLANTS

*The Material.*—The roots, stems and leaves were softened by moistening in a solution of formaldehyde (5 cc.) in water (500 cc.). Hand sections of the material were cut and examined under the microscope. Iodine (5 Gm.) in potassium iodide (10 Gm.) solution in water (100 cc.) was used for staining the sections for a study of the distribution of alkaloids in different tissues.

The alkaloids are similarly distributed in different tissues of both young and old plants. The alkaloids are present in their stems, in the medullary rays and in the outermost regions of the cell walls of the xylem. In the leaves, the sclerenchymatous hypodermis, the pericycle, the xylem of the vascular bundles, the mid-ribs and the veins passing through the mesophyll contain alkaloids. In both fibrous and main roots of the plants, alkaloids are most abundant in a ring of parenchymatous tissues around the vascular bundles, the sclerenchymatous pericycle and the hard bast of the phloem. The xylem elements and cells of the cortex show the presence of alkaloids (20). In all cases, the phloem in the leaves, stems and roots is free from alkaloids.

*Interpretation of the Results.*—Examination of the distribution of alkaloids throughout the various tissues reveals that the alkaloids are present in largest amounts (*a*) in parts of the plants which grow in absence of light, *e. g.*, roots, (*b*) in cortical tissues (the plant possesses a deep-seated phellogen and as such a large cortical area) (20) and (*c*) in tissues which are known chiefly to perform mechanical functions, *e. g.*, the sclerenchyma. The alkaloids are found to be stored mainly in dead and other cells, which take no part in metabolic activities and so are not drawn into metabolic changes, and thus the alkaloids accumulate from year to year; this accounts for the larger alkaloid content of older plants as shown previously.

### B. THE INFLUENCE OF FEEDING THE PLANTS WITH DIFFERENT FERTILIZERS

Several two- and four-year-old plants were examined for their alkaloidal content in root and stem

bark and then potted in well-washed sand and watered weekly with different culture solutions containing inorganic and organic nitrogenous compounds, phosphates, glucose, etc.

*Solution 1* contained calcium nitrate (0.075%), potassium nitrate (0.025%), magnesium sulfate (0.025%), potassium dihydrogen phosphate (0.025%) and ferric chloride in traces. *Solution 2* contained glucose (0.25%) mixed in solution 1. *Solution 3* contained ammonium nitrate (0.075%), calcium nitrate (0.025%) and magnesium sulfate, etc., in the same proportion as in solution 1. *Solution 4* contained potassium nitrate (0.025%) and glucose (0.25%) mixed in solution 3. *Solution 5* contained urea (0.1%), calcium phosphate (0.025%), magnesium sulfate (0.025%) and ferric chloride in traces. In *solution 6*, urea of solution 5 was replaced by asparagine (0.1%); the other ingredients were not changed. *Solution 7* contained potassium dihydrogen phosphate (0.025%) instead of urea of solution 5, together with other ingredients of the latter solution. *Solution 8* contained ammonium nitrate (0.05%), together with other ingredients of solution 1, except calcium nitrate, and *solution 9* contained ammonium nitrate (0.075%) and other ingredients of solution 5, except urea.

fed plants, as in the previous cases of the culture experiments with solution 2, showed an increase in the alkaloid content.

So far the effects of inorganic nitrogen as nitrates of calcium, potassium and ammonium have been mentioned. Now the results of feeding the plants, with sources of organic nitrogen as urea (solution 5) and asparagine (solution 6) are recorded. (See Table II (*e*) and (*f*)). A set of three plants six years of age was watered with solution 5, and a second set of three plants, of the same age, was watered with solution 6, for seven months from March till the end of September, 1942. During this period both sets of plants showed healthy growth and flowered. The alkaloidal content showed practically no increase in the first set of plants, whereas in the second set the increase in the alkaloid content was remarkable. The effect of urea on the plants was, therefore, more or less similar to that of ammonium nitrate with calcium nitrate (solution 3).

*Interpretation of the Results.*—The results of the culture experiments with nitrates alone did not show any increase in the alkaloid content of the plants, which may be due to the luxuriant growth of the plants, needing the utilization of all the carbohydrates and nitrates available for protein synthe-

TABLE I.—MEAN VALUES OF THE VARIATION OF THE TOTAL ALKALOIDAL CONTENT WITH AGE AND SEASON (GM. B, HI/100 GM. DRIED TISSUE)

| | 2-Year-Old Plants | | | 4-Year-Old Plants | | | A 14-Year-Old Plant | | |
|---|---|---|---|---|---|---|---|---|---|
| | Whole Root | Stem Bark | Stem | Whole Root | Stem Bark | Stem | Whole Root | Stem Bark | Stem |
| Dec.–Feb. | 0.339 | 0.217 | 0.143 | 0.406 | 0.347 | 0.244 | 0.652 | 0.494 | 0.341 |
| Mar.–May | 0.325 | 0.183 | 0.110 | 0.394 | 0.290 | 0.164 | 0.621 | 0.395 | 0.227 |
| June–Aug. | 0.184 | 0.112 | 0.045 | 0.210 | 0.156 | 0.074 | 0.321 | 0.220 | 0.089 |
| Sept.–Nov. | 0.223 | 0.129 | 0.053 | 0.271 | 0.210 | 0.091 | 0.431 | 0.275 | 0.118 |

(*a*) *Cultural Experiments with Various Sources of Nitrogen.*—The results of feeding two- and four-year old plants with calcium and potassium nitrates as sources of nitrogen (solution 1) for a year from March, 1939, to February, 1940, are given. The final estimations were done in March, 1940, and the control estimations in March, 1939.

The plants thrived well when fed with solution 1, containing the nitrates of calcium and potassium. New growth was observed during the rains in July, but the plants did not flower. The alkaloid content did not show any increase.

Three- and five-year-old plants were fed weekly with glucose together with the nitrates of potassium and calcium (solution 2) for a period of eight months, from the beginning of March until the end of October, 1941. The plants showed strong growth and two of the five-year-old plants flowered, after the rains, in September. Under normal conditions these plants do not flower at such an early age. The addition of glucose caused a definite increase in the alkaloid content of the various tissues. All these estimations were carried out during November, 1941, and control estimations in March, 1941.

Three five-year-old plants were fed weekly with solution 3, containing excess of ammonium nitrate, for a period of seven months, from September, 1941, to the end of March, 1942. They did not show any appreciable increase in the alkaloid content.

Another set of three plants was watered once a week with solution 4, containing glucose in addition to ammonium nitrate. They showed stronger growth compared with that of the first set of plants fed with solution 3, and they were found to endure the cold season during December and January much better than the former set of plants. The glucose-

sis. When the feeding solution contained both glucose and nitrates, there was an excess of them, which might have produced an excess of amino acids, after the synthesis of the required amount of protein. This excess of amino acids might have subsequently been used up in increased alkaloidal production.

The combination of ammonium nitrate and urea does not increase the alkaloidal content in the plants.

Asparagine, as the sole source of nitrogen, causes a very definite increase in the quantity of alkaloids in all tissues. This might be explained as either asparagine or aspartic acid is utilized by the plants directly in the synthesis of the alkaloids or asparagine yields aspartic acid which, in turn, might yield malic acid on deamination. This malic acid, obtained from an external source, might cause an increased production of the alkaloids, which naturally would have been obtained from amino acids and carbohydrates of the plants.

The increase in the amount of the alkaloids, which is enhanced by the supply of an external source of nitrogen and organic acids, is independent of growth. During the period of active growth under normal conditions there is a regular increase in the alkaloid content from year to year.

(*b*) *Cultural Experiments with Different Solutions, with a View to Starve the Plants.*—Six of the six-year-old plants were watered with a phosphate solution containing no nitrogen compounds (solution 7) for a period of nine months, from March to November, 1942. No growth took place and no fresh leaves appeared even during or after the rains in September. The plants appeared to be drooping.

A second series of culture experiments was done with a set of six plants, five years old. The plants were fed for six months, from March to July, 1942,

with a solution of nitrates of potassium and ammonium, but containing no calcium salt (solution 8). During that period, no new growth was observed; four of the plants became gradually diseased and died.

A third series of experiments upon a set of three plants, five years old, was made with a solution containing both ammonium nitrate and calcium phosphate, but no potassium salt (solution 9). The results were more or less similar to those of plants watered with solution 8, which contained no calcium salt. No new growth was observed, and the alkaloidal content of the plants remained practically unchanged after the period of experiment from March to July, 1942. (For the results of estimations of the alkaloid content see Table II (*g*), (*h*) and (*i*).)

TABLE II.—CULTURE EXPERIMENTS WITH DIFFERENT SOLUTIONS. (THE ALKALOID CONTENT—GM. B, HI/100 GM. DRIED TISSUE)

| | Before Experiment | | After Experiment | |
|---|---|---|---|---|
| | Whole Root | Stem Bark | Whole Root | Stem Bark |
| (*a*) Culture Experiments with Solution 1 | | | | |
| Plant aged 2 yr. | 0.318 | 0.200 | 0.305 | 0.192 |
| | 0.325 | 0.214 | 0.321 | 0.187 |
| | 0.309 | 0.187 | 0.310 | 0.175 |
| Av. | 0.318 | 0.203 | 0.312 | 0.184 |
| Plant aged 4 yr. | 0.401 | 0.290 | 0.392 | 0.285 |
| | 0.389 | 0.275 | 0.385 | 0.274 |
| | 0.391 | 0.284 | 0.299 | 0.274 |
| Av. | 0.393 | 0.283 | 0.358 | 0.277 |
| (*b*) Culture Experiments with Solution 2 | | | | |
| Plant aged 3 yr. | 0.235 | 0.165 | 0.258 | 0.230 |
| | 0.231 | 0.158 | 0.250 | 0.219 |
| | 0.229 | 0.143 | 0.261 | 0.223 |
| Av. | 0.232 | 0.155 | 0.256 | 0.224 |
| Plant aged 5 yr. | 0.283 | 0.220 | 0.354 | 0.310 |
| | 0.275 | 0.205 | 0.346 | 0.301 |
| | 0.280 | 0.196 | 0.341 | 0.319 |
| Av. | 0.279 | 0.207 | 0.347 | 0.310 |
| (*c*) Culture Experiments with Solution 3 | | | | |
| Plant aged 5 yr. | 0.283 | 0.219 | 0.285 | 0.200 |
| | 0.274 | 0.205 | 0.269 | 0.201 |
| | 0.275 | 0.185 | 0.271 | 0.195 |
| Av. | 0.277 | 0.203 | 0.275 | 0.199 |
| (*d*) Culture Experiments with Solution 4 | | | | |
| Plant aged 5 yr. | 0.279 | 0.222 | 0.403 | 0.335 |
| | 0.265 | 0.211 | 0.281 | 0.400 |
| | 0.280 | 0.220 | 0.397 | 0.386 |
| Av. | 0.275 | 0.224 | 0.363 | 0.375 |
| (*e*) Culture Experiments with Solution 5 | | | | |
| Plant aged 6 yr. | 0.411 | 0.347 | 0.405 | 0.352 |
| | 0.453 | 0.359 | 0.455 | 0.346 |
| | 0.434 | 0.329 | 0.432 | 0.311 |
| Av. | 0.433 | 0.345 | 0.431 | 0.336 |
| (*f*) Culture Experiments with Solution 6 | | | | |
| Plant aged 6 yr. | 0.415 | 0.339 | 0.548 | 0.542 |
| | 0.427 | 0.358 | 0.555 | 0.465 |
| | 0.434 | 0.331 | 0.521 | 0.463 |
| Av. | 0.425 | 0.343 | 0.541 | 0.490 |
| (*g*) Culture Experiments with Solution 7 | | | | |
| Plant aged 6 yr. | 0.400 | 0.339 | 0.408 | 0.331 |
| | 0.405 | 0.340 | 0.403 | 0.331 |
| | 0.395 | 0.327 | 0.387 | 0.310 |
| | 0.401 | 0.346 | 0.395 | 0.330 |
| | 0.389 | 0.337 | 0.395 | 0.351 |
| | 0.402 | 0.316 | 0.393 | 0.324 |
| Av. | 0.399 | 0.338 | 0.397 | 0.330 |
| (*h*) Culture Experiments with Solution 8 | | | | |
| Plant aged 5 yr. | 0.275 | 0.214 | 0.298 | 0.235 |
| | 0.283 | 0.196 | 0.303 | 0.291 |
| Av. | 0.279 | 0.205 | 0.300 | 0.263 |
| (*i*) Culture Experiments with Solution 9 | | | | |
| Plant aged 5 yr. | 0.269 | 0.209 | 0.271 | 0.201 |
| | 0.277 | 0.219 | 0.282 | 0.211 |
| | 0.283 | 0.201 | 0.273 | 0.207 |
| Av. | 0.276 | 0.210 | 0.275 | 0.206 |

*Interpretation of the Results.*—The alkaloidal content did not decrease even when the plants were fed with nonnitrogenous salts (solution 7). During these culture experiments the plants had necessary conditions to utilize the available nitrogen present in the alkaloids. Since there was no appreciable decrease in the alkaloidal content, it can be safely concluded that the plant is unable to make use of the nitrogen of the stored alkaloid, even under favorable conditions. In other words, the alkaloid in the *Mahonia* plants is not a stored food, to be used up by the plant when required, but a waste product. The slight increase in the alkaloid value in stray cases would indicate that the plant is drawing upon the excess of nitrogen present in forms other than the alkaloids, and even in conditions which are rather unfavorable to the building up of the alkaloids, the production of the alkaloids still continues. These observations remind one of those of Mothes (16), who showed that nicotine was still synthesized in conditions unfavorable to the growth of the *Nicotiana* plants.

Since calcium is an essential factor in the mechanism of protein synthesis and in building and development of the cell walls, the death and stunted growth of some of the plants in the absence of calcium salts (solution 8) can easily be accounted for. The appreciable increase in alkaloid value shows that protein degradation proceeds normally during the period of experiment, but the external nitrogen or the nitrogen reserves are not able to regenerate protein, because of calcium deficiency.

It is well known that the plants, not getting any potassium salts, usually show carbohydrate deficiency. The stationary alkaloid values in the last case when the plants are fed with solution 9, may be due to the carbohydrate deficiency in proportion to the amount of available nitrogen.

### C. THE EFFECT OF ETIOLATION

Several two-year-old plants were potted in sand and kept in complete darkness for three months and watered weekly with solution 1.

These plants produced yellow shoots, which were found to contain cells rich in alkaloids. Plant No. 3 had a young leaf cone before placing in darkness; the cone showed rapid growth and the portion growing in darkness showed a greater concentration of alkaloids. Plant No. 4 was exposed to light after keeping it in complete darkness for a period of three months. The yellow color of the etiolated shoots gave place to green color of chlorophyll. The amount of the alkaloids decreased and alkaloids are only found in the lower portion of the shoots; upper portion showed practically no trace of alkaloids after a one-month exposure to light.

*Interpretation of the Results.*—It is evident that the figures in Table III show that during growth of the plants in darkness, the amount of the alkaloids increases in a marked degree, in all parts of the plants. The etiolated shoots also show a much greater concentration in relation to the age of the tissue. Exposure of plant No. 4 to light shows that light plays an important role in the production and location of the alkaloids. All these results are in good agreement with those of Ripert (19) working on the atropine and hyoscyamine contents of *Atropa*, of Cromwell (6) for the berberine content of *Berberis* and of Weevers (4) for the alkaloid content of etiolated *Ricinus* seedlings. And it is found in plant No. 3 that the alkaloidal content is greater in the part of the tissue which developed in darkness. These give a strong support to the conclusion that the alkaloids are synthesized in the growing etiolated tissues. The high concentration of the alkaloids in the etiolated shoots in plant No. 4 is due to the synthesis of the alkaloids in the growing region and not to translocation as the portion of the shoot growing later, in the presence of light, showed practically no alkaloids. These experiments, as a whole, show that an increase of the protein breakdown leads to an increase in the alkaloidal concentration. Thus they point to the close connection between the protein breakdown and the synthesis of the alkaloids in *Mahonia* plants.

TABLE III.—EFFECTS OF ETIOLATION

(The Alkaloidal Content—Gm. B, HI/100 Gm. Dried Tissue)

| | Before Experiment | | After Experiment | | |
|---|---|---|---|---|---|
| | Whole Root | Stem Bark | Whole Root | Stem Bark | Etiolated Shoots |
| Plant aged 2 yr. No. 1 | 0.305 | 0.181 | 0.617 | 0.270 | 0.555 |
| No. 2 | 0.316 | 0.211 | 0.651 | 0.321 | 0.584 |
| No. 3 | 0.318 | 0.212 | 0.648 | 0.315 | 0.701 |
| No. 4 | 0.317 | 0.219 | 0.631 | 0.303 | 0.674 |

TABLE IV.—RINGING EXPERIMENTS

| | Stem below Ring | Stem above Ring | Etiolated Shoots |
|---|---|---|---|
| Young plants which had not yet flowered | 0.181 | 0.075 | .. |
| | 0.117 | 0.056 | .. |
| | 0.169 | 0.092 | .. |
| Av. | 0.155 | 0.074 | .. |
| Plant age 3 years potted in sand, kept in darkness for 3 mo. and watered weekly with solution 1 | 0.151 | 0.111 | 0.73 |
| | 0.167 | 0.141 | 0.81 |
| | 0.123 | 0.76 | .. |
| Av. | 0.147 | 0.109 | .. |

### D. RINGING EXPERIMENTS

These experiments were made for a further observation on whether alkaloids are actually synthesized in the region of growth of the etiolated tissue or accumulated due to translocation. A ring of bark, one inch thick, was removed from the main stem and the ring was painted with grease and then covered with a bandage. At the end of six months the alkaloidal concentrations of the stems above and below the ring were determined. The results are shown in Table IV.

*Interpretation of the Results.*—In most of the ringed plants, off-shoots on the stem grew below the ring. No growth took place above the ring. The figures in Table IV show a high value for the alkaloidal concentration below the ring in both normal and etiolated plants. Etiolated shoots rich in alkaloids appeared only below the ring. The accumulation of alkaloids in the shoots can only be explained as due to either synthesis in the region of growth or translocation upward from the stem and root. The effects of etiolation on plants Nos. 3 and 4 (see Table III) together with the ringing experiments on etiolated plants (see Table IV) show definitely that alkaloids are synthesized in the growing etiolated tissues.

## SUMMARY

1. Experiments upon *Mahonia nepalensis* DC. (*Berberis nepalensis* Spreng.) have been made to throw light on: (*a*) the histological distribution of alkaloids in various tissues of the plant, (*b*) the variation in the alkaloidal content of the plants with age and season, (*c*) the effect of feeding the plants with various nitrogenous and nitrogen-free culture solutions, (*d*) the changes brought about by etiolation, and (*e*) by ringing of the bark.

2. It is found that in *M. nepalensis*, the alkaloids, umbellatine and neprotine, appear to be waste products of metabolism, since they are stored mainly in dead and other cells which take no part in metabolic activities and so are not drawn at all into metabolic changes.

3. Synthesis of the alkaloids takes place during the period of active growth. The alkaloids accumulate from year to year and their quantity does not decrease during the winter.

4. The feeding with asparagine leads to an increase in the alkaloid content of the plants. With ammonium salts and inorganic nitrates, glucose has to be applied to increase the alkaloid content. On feeding with nitrogen-free culture solutions, the plants do not show a decrease in the alkaloid content. All these point to a connection between the production of alkaloids and the decomposition of protein. It is suggested that the synthesis of alkaloids is brought about by the condensation of sugars and the degradation products of protein.

5. On ringing the bark out, new shoots

appear only below the ring. In etiolated plants, when the bark is ringed out, shoots rich in alkaloids sprout. The plants, when etiolated, also produced shoots rich in alkaloids. These experiments show that an increase of the protein breakdown leads to an increase in the alkaloidal concentration, suggesting a close connection between the protein breakdown and the synthesis of alkaloids in *Mahonia* plants.

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# A Chemical Study of *Mahonia nepalensis* DC. (*Berberis nepalensis Spreng.*)*

By R. Chatterjee†

Although several species of the genus *Berberis*, family *Berberidaceae*, have been more or less thoroughly investigated (1, 3), few attempts have been made to examine the Himalayan species of *Berberis*. It has been found that berberine and associated alkaloids are present in the European species of *Berberis* (1), but both *B. umbellata* Wall. (2) and *B. insignis* Hook. f. (3), two of the Himalayan species of *Berberis*, showed the presence of the same alkaloid, umbellatine, $C_{21}H_{21}O_8N$. Hoping that the examination of other species might lead to interesting results, investigations were started on *Berberis* growing in the Himalayas, and in this paper the results of the chemical examination of *Mahonia nepalensis* DC. have been described. Two alkaloids were isolated; one of them was found to be identical with umbellatine, the alkaloid found in both *B. umbellata* Wall. and *B. insignis* Hook. f. Table I shows the different alkaloids isolated from *Mahonia*.

*M. nepalensis* grows abundantly in the temperate regions of the Himalayas, at an altitude of 4000 to 8000 feet (6). It grows wild in Darjeeling (alt. 6500 feet). A sample collected locally was kindly identified by Mr. S. N. Basu, Curator, the Lloyd Botanic Garden, Darjeeling. The total alkaloidal content was estimated in different parts of both old and young plants which had not yet bloomed and it was found that the roots of the old plants had the greatest amount of alkaloids. Further gravimetric assays with changes of seasons showed that the largest possible amount of alkaloids can be isolated from the roots of old plants in the winter.

The roots of the plants were collected during the winter months, January to March, and umbellatine, $C_{21}H_{21}O_8N$, and a garnet-red crystalline alkaloid, to which the formula $C_{19}H_{21}O_6N$ is provisionally given, were isolated. The composition and properties of the latter alkaloid are not in agreement with those of any known alkaloid of the genus *Berberis* or *Mahonia*. Indeed, evidence shows that it is a new alkaloid, and the name neprotine is proposed for it.

Neprotine decomposes above 200° C. without melting, and is optically inactive. It forms elongated prisms on slow crystallization. It is easily soluble in alcohol and water, very slightly soluble in chloroform but insoluble in light petroleum ether, ether, ethyl acetate and acetone. Neprotine forms

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an orange-red hydrochloride, an orange-colored picrate, a yellow-colored sulfate and a yellow nitrate. It loses weight amounting to 3.5 molecules of water and retains one molecule of water of crystallization even on drying *in vacuo* over $P_2O_5$ at 100° C.

## EXPERIMENTAL

*Isolation and Separation of Umbellatine and Neprotine.*—For isolating the alkaloids, 1 Kg. of powdered air-dried root was extracted by means of a Soxhlet extractor with alcohol, until the siphoned solution was faintly yellow. The alcohol of the extract was removed and the brown residue treated with 500 cc. of water and filtered. The filtrate was treated with hydrochloric acid to give a 1% solution of the aqueous extract and allowed to stand overnight. Crystals of a yellow hydrochloride separated and were filtered and well washed until the washings were yellow in color. The brown-colored filtrate was concentrated on a water bath to 400 cc. and filtered twice, and this solution being left overnight, a second crop of yellow crystals appeared. The solution was decanted carefully and evaporated partially and was left in a crystallizing dish. After two days red granules appeared and the dish was left undisturbed for a week till the quantity of granules stopped increasing. The red granules were filtered, well washed and dried. The filtrate was allowed to evaporate at the ordinary temperature, but no crystals appeared; eventually a very sticky brown mass was left behind which could not be crystallized. The brown mass was treated according to the methods followed by Kondo and Tomita (7) and Orekhov (8) in isolating *Berberis* alkaloids, but with little success.

Another 1 Kg. of the powdered root was extracted during the cold season, at a room temperature of 13° C., the sticky brown mass after the removal of alcohol was allowed, without treatment with water, to stay overnight, when a crop of yellow granules separated, and was taken out with a spatula. The sticky mass was then divided into three portions and each portion was treated with 200 cc. of water and then warmed with hydrochloric acid to give a 1% solution of the aqueous extract and allowed to stand for three days, when red granules were precipitated and filtered off. The filtrate was then concentrated on the water bath and allowed to stand, when a crop of yellow needles crystallized out.

*Treatment of the Yellow Hydrochloride.*—The well washed yellow hydrochloride obtained in the form of needles and granules was recrystallized from hot water containing a few drops of hydrochloric acid; the crystals were filtered and the filtrate on concentration yielded a very small quantity of the red hydrochloride mixed with the yellow one. The former was separated by fractional crystallization from 50% alcohol. The crystals of the yellow hydrochloride were purified by repeated crystallization from hot alcohol as shining yellow silky needles. (Yield, 4.8 Gm./Kg. of dried roots.) The crystals decomposed without melting.

*Preparation of the Free Base and Identification of Umbellatine.*—To prepare the free base, the hydrochloride (5 Gm.) was dissolved in hot water, made alkaline with diluted solution of sodium carbonate, whereupon the yellow color of the solution changed to dark brown, and on cooling, yellow silky needles of the free alkaloid were obtained. They were purified by repeated crystallizations from hot water and finally from hot alcohol. (Yield, 4.4 Gm.) The alkaloid melted at 205–207° C. (decomp.); mixed with umbellatine, it melted at 204–206° C. (decomp.). The results of combustion analysis conform to the formula $C_{21}H_{21}O_5N$.

*Anal.*—Calcd. for $C_{21}H_{21}O_5N.{}^1/_2H_2O$: C, 59.4; H, 5.2; N, 3.3. Found: C, 59.2; H, 5.4; N, 3.2.

That this yellow alkaloid is umbellatine is further supported by the comparison of the properties of its derivatives with those of umbellatine derivatives.

The picrate of the alkaloid melts at 231° C. (decomp.). Mixed with umbellatine picrate, it melts at 231° C. (decomp.).

*Anal.*—Calcd. for $C_{21}H_{21}O_5N.C_6H_3O_7N_3$: N, 8.7. Found: N, 8.9.

The sulfate of the alkaloid melts at 271–273° C. (decomp.). Mixed with umbellatine sulfate, it melts at 273–274° C. (decomp.).

*Umbellatine Nitrate.*—The so-called nitroso derivative of umbellatine is afterward found to be the nitrous acid salt of umbellatine (9). Umbellatine nitrate melts at 265–267° C. (decomp.). Mixed with the nitrous acid derivative of the alkaloid, it melts at 265–267° C. (decomp.).

*Anal.*—Calcd. for $C_{21}H_{21}O_5N.HNO_2$: N, 6.7. Found: N, 6.3.

*Treatment of the Red Hydrochloride.*—The small quantity of the crude red hydrochloride was purified

TABLE I.—ALKALOIDS OF *Mahonia* SPECIES

| Species | Alkaloids | Molecular Formula | References |
|---|---|---|---|
| *M. aquifolium* Nutt. | Berberine | $C_{20}H_{19}O_5N$ | (4) |
| (*B. aquifolium* Pursh) | Berbamine | $C_{37}H_{40}O_6N_2$ | |
| | Oxyacanthine | $C_{37}H_{40}O_6N_2$ | |
| *M. philippensis* Nutt. | Berberine | | (5) |
| | Jatrorrhizine | $C_{20}H_{20}O_4NI$ | |
| | Shobakunine | (the iodide) | |
| *M. nepalensis* DC. | Umbellatine | $C_{21}H_{21}O_5N$ | |
| (*B. nepalensis* Spreng.) | Neprotine | $C_{19}H_{21}O_6N$ | |

TABLE II.—THE TOTAL ALKALOID OF *M. nepalensis*

| | Whole Root | Stem-bark | Stem |
|---|---|---|---|
| (*a*) In old plants (— Gm./100 Gm. air-dried parts) | | | |
| Dec.–Feb. | 0.498 | 0.377 | 0.261 |
| Mar.–May | 0.474 | 0.302 | 0.173 |
| June–Aug. | 0.246 | 0.168 | 0.068 |
| Sept.–Nov. | 0.330 | 0.210 | 0.090 |
| (*b*) In young plants (— Gm./100 Gm. air-dried parts) | | | |
| Dec.–Feb. | 0.310 | 0.265 | 0.186 |
| Mar.–May | 0.301 | 0.221 | 0.125 |
| June–Aug. | 0.160 | 0.119 | 0.057 |
| Sept.–Nov. | 0.207 | 0.160 | 0.070 |

by repeated crystallization from hot water and finally from 50% alcohol; orange-red colored elongated prisms appeared on slow crystallization. They charred without melting. (Yield, 0.2 Gm./Kg. of dried roots.)

*Preparation of the Free Base, Neprotine.*—The purified orange-red hydrochloride, neprotine hydrochloride (0.2 Gm.), was dissolved in hot water and a diluted solution of sodium carbonate was added until alkaline, when the brown color of the solution changed to blood-red, and on cooling, garnet-red needles of the free alkaloid were obtained. They were purified by repeated crystallizations from alcohol; garnet-red colored elongated prisms were obtained on slow crystallization, which charred without melting. (Yield, 0.18 Gm.)

The quantity of umbellatine and the new alkaloid, neprotine, vary considerably with seasons. Table III shows the quantity in Gm. of umbellatine and neprotine obtained from 1 Kg. of air-dried roots of the plant collected in different seasons.

TABLE III.—ALKALOIDS OF *M. nepalensis* (GM./KG. AIR-DRIED ROOTS)

| | Dec.–Feb. | March–May | June–Aug. | Sept.–Nov. |
|---|---|---|---|---|
| Umbellatine | 4.8 | 4.6 | 2.50 | 3.10 |
| Neprotine | 0.2 | 0.2 | 0.09 | 0.15 |

Neprotine reacts with alkaloidal reagents, as shown in Table IV.

TABLE IV.—REACTIONS OF NEPROTINE WITH ALKALOIDAL REAGENTS

| | |
|---|---|
| Conc. sulfuric acid | Dissolves with a deep yellow color |
| Fröhde's reagent | Dissolves with a yellow color which changes to greenish yellow and eventually to sherry-red |
| Mandelin's reagent | Yellow color changing to green on long standing |
| Mayer's reagent | A yellow precipitate |
| Picric acid | A light orange precipitate |
| Potassium iodide solution (10%) | An orange-yellow precipitate |
| Wagner's reagent | A yellow precipitate |

The results of the analyses of neprotine and its salts did not show a definite molecular formula for it. The formula $C_{19}H_{21}O_6N$ is given to neprotine, provisionally, as it is found to tally with most of the analytical results of neprotine and its salts.

*Anal.*—Calcd. for $C_{19}H_{21}O_6N\,H_2O$: C, 60.48; H, 6.10; N, 3.71. Found: C, 60.46; H, 6.08; N, 3.91.

*Neprotine Hydrochloride.*—Neprotine hydrochloride is prepared by the usual method, as garnet-red elongated prisms.

*Anal.*—Calcd. for $C_{19}H_{21}O_6N.HCl.1\frac{1}{2}H_2O$: Cl, 8.4; loss of water, 6.4. Found: Cl, 8.5; loss of water, 7.0. Calcd. for anhydrous $C_{19}H_{21}O_6N$, HCl: N, 3.53. Found: in a vacuum dried sample: N, 3.5.

*Neprotine Chloroplatinate.*—Neprotine chloroplatinate is prepared by the usual method as a yellow amorphous powder, which could not be crystallized from an organic solvent or water. It decomposed without melting.

*Anal.*—Calcd. for $(C_{19}H_{21}O_6N)_2H_2PtCl_6$: Pt, 17.28. Found: Pt, 17.58, 17.22.

*Estimation of the Total Alkaloid of M. nepalensis.*—The method of isolation of the alkaloids of *M. nepalensis* was followed. Ten grams of the air-dried, well-powdered material was extracted with alcohol and the bases were precipitated as hydrochlorides, purified, and the free bases liberated were weighed on a tared filter paper.

## SUMMARY

1. *Mahonia nepalensis* DC. (also called *Berberis nepalensis* Spreng.) contains about 0.48% of umbellatine, $C_{21}H_{21}O_8N$, the alkaloid present in the Himalayan species of *Berberis, B. umbellata* Wall. and *B. insignis* Hook. f., and 0.02% of a hitherto unknown red alkaloid, neprotine, to which a provisional formula, $C_{19}H_{21}O_6N$, is given. Some of the physical properties and the hydrochloride and chloroplatinate of neprotine have been described.

2. The seasonal variation of the total alkaloidal contents of different parts, roots, stem-barks and stems, of the plant as well as those of individual alkaloids, umbellatine and neprotine, are recorded. It is found that the highest concentration of alkaloids, *e. g.*, umbellatine, 0.48% and neprotine, 0.02%, is in the roots of the old plants, during the winter months.

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# The Estimation of Small Amounts of Lead in National Formulary Preparations by the Dithizone Method*

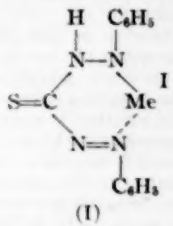
*By Celesta A. Steinmetz and Melvin W. Green*†

Both the United States Pharmacopœia and the National Formulary use an omnibus type of heavy metals test as one of the means of limiting the degree of impurity permitted in pharmaceuticals. Unfortunately, however, there are many cases where the test is inadequate in that it is not specific for any one metal and the color of the solution is often modified by the hydrogen sulfide even in the absence of significant quantities of heavy metals. Furthermore, the heavy metals test is useless in colored solutions. Salts of heavy metals such as the iron salts used in medicine cannot be tested for the presence of foreign heavy metals at the present time. To conduct such a test, it is obvious that it would be necessary to remove completely the cation of the salt to be tested without removing the heavy metal cations present as impurities. In this connection, in this laboratory significant quantities of lead were added to a soluble iron salt, the iron precipitated with ammonia water and the filtrate treated with hydrogen sulfide. There was no darkening of the filtrate indicating that the added lead had been adsorbed on the finely divided ferric hydroxide.

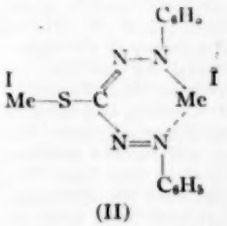
Furthermore, it must be borne in mind that lead is toxic. Lead is slowly absorbed from the gastrointestinal tract, but constant accumulation of lead is frequently sufficient to result in lead poisoning. Lead is taken in by the consumption of food and from the environment. The average daily incidental exposure is probably from 0.2 to 0.4 mg. of lead. The excretion of lead more or less keeps pace with the intake unless one is exposed to an excessive amount of lead (1). A drug such as soluble ferric pyrophosphate, which has an average daily dose of 0.75 Gm. and has a lead content of 290 p. p. m., will increase the daily exposure to lead by 0.218 mg. which is about equal to the normal daily exposure to environmental lead.

It is for these reasons that it is believed that the introduction of a specific test for lead in the monographs of the National Formulary and the United States Pharmacopœia would be highly desirable. The dithizone method for the microdetermination of lead offers such a possibility.

Diphenylthiocarbazone (dithizone) was introduced into analytical practice by Helmut Fischer (7), and may be used for determining some fifteen metals, but by careful control of conditions it can be made specific for lead. There seems to be a stoichiometric relationship between the organic reagent and the metal due to the formation of an inner complex salt which is soluble in organic solvents. These coördinate compounds can be formed due to the fact that dithizone is capable of undergoing keto-enol tautomerization (7). Two types of inner complexes can thus be formed depending on the tautomer present, the reaction conditions and the valence of the metal. In the keto form the metal replaces the H of the imide group and forms a coördinate bond with the other nitrogen. The enol forms, II, III, contain twice as much metal as the keto form, I, for in addition to this linkage, the H of the sulfhydryl group is replaced with metal. For divalent metals both couplings are combined in one. These compounds are illustrated in the following formulas:



(I)



(II)

(III)

It is thought that it is the inner complex nature of these compounds which is responsible for the solubility in organic solvents. The keto forms are stable in acid and neutral solutions while the enol forms are

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stable only in alkali. The lead complex is believed to exist only in the keto form.

The dithizone test is not new, for it is used in foods by the Food Division of the Federal Food and Drug Administration (2). Furthermore, members of the staff of the Kettering Laboratory of Applied Physiology and others have done extensive work with the dithizone test on biological materials (3, 4). A modification of the dithizone method has been successfully used for the determination of lead in cosmetics (5), and it has also been adapted to the determination of lead in substances stored in collapsible lead tubes (6). At present the National Formulary (14) has a test which determines the limit of lead by comparing the number of extractions required for the substance being tested to the number necessary for the extraction of a standard containing the stipulated amount of lead adjusted for the quantity of substance tested. The end point in the extraction is the point at which the chloroform layer has turned from a pinkish color to a purple. This test was developed primarily for the determination of lead in Pectin and Cherry Juice.

## EXPERIMENTAL

The chemical to be tested is usually dissolved in a dilute nitric acid solution to which are added varying quantities of solutions of ammonium citrate, potassium cyanide and hydroxylamine hydrochloride. The proper quantities of these solutions depend upon the chemical nature of the substance being analyzed. The ammonium citrate solution decreases the solubility of dithizone in dilute ammoniacal solution (8) and prevents the precipitation of phosphates and hydroxides in an alkaline media (9). The potassium cyanide ties up many metals and thus prevents their interfering with the lead-dithizone reaction (8). The hydroxylamine hydrochloride provides a reducing environment which aids in preventing the oxidation of dithizone. Furthermore, it prevents interference from ferric iron by keeping the iron in a reduced state (10).

At this point the mixture is made just alkaline to phenol red with ammonium hydroxide, yielding the optimum *p*H about 8.5, to extract the lead from the aqueous solution into the chloroform solution. The *p*H at this point is important because, as has been shown in previous work, only at the proper *p*H will all the lead combine with the dithizone and be completely extracted from the aqueous phase (9). If the *p*H is too low, some of the lead remains in the aqueous solution and the final results are not correct.

Dithizone, which is dissolved in chloroform, is added to the aqueous alkaline solution and shaken for one minute. Long shaking is not necessary for the complex is quickly formed and further shaking increases the possibility of loss of the lead (8). The lead present forms a complex with the dithizone which is soluble in the chloroform and gives a green, purplish blue, reddish purple or a red color to the chloroform, depending upon the amount of lead reacting with the dithizone. If the chloroform solution has any purplish or reddish color, it is drawn off and the aqueous solution is shaken with portions of dithizone solution until the dithizone solution retains its original green color. A slight excess amount of dithizone solution must be used to insure complete extraction of lead from the aqueous solution.

To the combined dithizone extractions is added a dilute nitric acid solution of a definite quantity and strength. This dilute nitric acid solution decomposes the lead dithizone complex and extracts the lead but leaves the free dithizone (8). The chloroform solution of the lead-free dithizone is discarded and a definite amount of ammonium cyanide solution is added to the dilute nitric acid solution containing the lead. The ammonium cyanide makes the solution alkaline and the cyanide buffers the solution so that slight variations in acid strength have no effect (9). The color developed is compared with the colors developed by treating standard lead solutions in the same manner. The colors range from green through mixed proportions of green and red giving purplish blue and purplish red hues or a bright red color.

Any pressure that is formed from shaking together the aqueous and chloroform solution should be released by removing the stopper of the separatory funnel and not by opening the stopcock in order to avoid blowing the solution into the funnel stem (11).

*Reagents* (11).—*Ammonium Hydroxide:* Reagent ammonium hydroxide is used for the test. If contaminated with lead it must be rendered lead-free by distillation.

*Ammonium Citrate Solution:* Dissolve 40 Gm. of reagent citric acid in 100 cc. of distilled water and make alkaline to phenol red with ammonium hydroxide. The solution is deleaded by shaking with small portions of dithizone solution until the dithizone retains its original green color.

*Potassium Cyanide Solution:* Dissolve 50 Gm. of potassium cyanide in sufficient distilled water to make 100 cc. Remove the lead by shaking with portions of the extraction dithizone solution. Part of the dithizone remains in the aqueous phase but can be removed, if desired, by washing with chloroform. The strong potassium cyanide solution is then diluted to a concentration of 10 Gm. in 100 cc.

*Hydroxylamine Hydrochloride Solution:* Dissolve 20 Gm. of hydroxylamine hydrochloride in sufficient distilled water to make about 65 cc. and add a few drops of thymol blue *p*H indicator solution. Add concentrated ammonium hydroxide until a yellow color appears. An approximately 4% solution of sodium diethyldithiocarbamate is added in sufficient quantity to combine with all the lead and to leave an excess reagent. After a few minutes completely extract with chloroform. This point is reached when a portion of the chloroform extract is shaken with a dilute solution of a copper salt and no yellow color appears in the chloroform layer. Hydrochloric acid is added to the solution until the indicator turns pink and distilled water is added to make the final volume 100 cc.

*Dithizone:* The dithizone prepared by the Eastman Kodak Company is satisfactory for all dithizone solutions without further purification.

*Dithizone Extraction Solution:* Dissolve 30 mg. of dithizone in a liter of chloroform and add a few cubic centimeters of alcohol. The amount of dithizone to be used for one day is shaken with about 100 cc. of 1% nitric acid just before use. Store the solution in a refrigerator (12).

*Standard Dithizone Solution:* This solution is one-third the strength of the dithizone extraction solution (10 mg. in 1000 cc.). It is kept in a glass-stoppered Pyrex bottle which is wrapped in heavy paper or kept in a wooden box and stored in the refrigerator (12).

*Ammonium-Cyanide Mixture:* To 20 Gm. of potassium cyanide add 150 cc. of ammonium hy-

droxide (specific gravity 0.9) or its equivalent and make to 1000 cc. with distilled water.

*Nitric Acid Solution, 1%:* Dissolve 10 cc. of nitric acid in enough distilled water to make a liter.

*Stock Lead Solution:* Dissolve 0.1599 Gm. of lead nitrate in 1000 cc. of 1% nitric acid solution.

*Standard Lead Solution:* Dilute 10 cc. of the stock lead solution to one liter with 1% nitric acid solution.

*Chloroform Recovery:* Used dithizone solution is stored in a Pyrex glass-stoppered bottle containing a few cubic centimeters of alcohol. Each liter of used dithizone is washed with about 200 cc. of distilled water and two 100-cc. portions of concentrated sulfuric acid. Five cubic centimeters of alcohol is added and it is allowed to stand overnight in the dark over lump calcium oxide. It is distilled over lump calcium oxide from an all glass connected still into a Pyrex bottle containing about 10 cc. of alcohol.

All glassware should be washed with nitric acid (1 in 1) and distilled water to avoid even a slight contamination of lead. All solutions should be stored in Pyrex, glass-stoppered bottles.

*Size of Sample.*—The size of the sample is governed by the amount of lead contamination present and therefore varies from a few hundredths of a gram to 10 Gm. If the sample tested contains a small amount of lead, the sample is large and is weighed out directly. If the lead contamination is large, the sample tested is small and an aliquot of the sample is taken. A convenient size sample is one that contains approximately 5 $\gamma$ of lead. The solubility of the chemical in water, dilute nitric acid or in alkaline solutions may also affect the size of the sample.

*Preparation of Sample.*—A sample (containing less than 10 $\gamma$ of lead) of most chemicals is dissolved in a few cc. of nitric acid (1 in 1) and distilled water, boiled and cooled. If the entire sample is used, it is transferred directly into the separatory funnel. If only a portion is to be used, it is made to a definite volume and an aliquot is taken. There are some exceptions to this general procedure as follows:

1. Lithium Bromide. The sample is dissolved in 3 cc. nitric acid (1 in 1) and a few cubic centimeters of distilled water and boiled until the brown color has disappeared and the solution is clear.

2. Lithium Carbonate. The nitric acid (1 in 1) is added slowly and with constant agitation.

3. Soluble Ferric Phosphate, Soluble Ferric Pyrophosphate, Ammonium Bromide, Ammonium Iodide, Solution of Ferric Chloride. These solutions are not heated.

4. Red Ferric Oxide, Yellow Ferric Oxide. The sample is dissolved in 8 cc. of hydrochloric acid by boiling. One cubic centimeter of nitric acid is added and the solution again boiled. Cool and make to volume with distilled water.

5. Saccharated Ferric Oxide, Saccharated Ferrous Carbonate. Ignite sample for two hours at 480° C. taking care to see that the temperature never rises above a dull red heat. Cool and dissolve in 3 cc. of hydrochloric acid and 1 cc. of nitric acid by warming the solution.

6. Iron. The sample is dissolved in 10 cc. of hydrochloric acid (1 in 1) by heating gently. Carefully add 3 cc. of nitric acid (1 in 1). Cool and make up to volume with distilled water.

7. Propylene Glycol. The sample is pipetted directly into a separatory funnel containing nitric acid (1 in 1) and a few cubic centimeters of distilled water.

8. Sucrose Octa Acetate. Add 1 cc. of nitric acid and ignite gently at a temperature not exceeding a dull red heat, adding a few drops of dilute nitric acid (1 in 1) from time to time. Dissolve the residue in 3 cc. of nitric acid (1 in 1) and a few cubic centimeters of distilled water; wash carefully into the separatory funnel with distilled water.

Table I shows the amount of the reagents to be used in each sample tested.

TABLE I.—AMOUNTS OF REAGENTS USED IN ESTIMATION OF LEAD

| Chemical | Nitric Acid (1 in 1), Cc. | Ammonium Citrate, Cc. | Potassium Cyanide, Cc. | Hydroxylamine Hydrochloride, Cc. |
|---|---|---|---|---|
| Alum. Sulf. | 3 | 3 | 1 | 0.5 |
| Ammon. Bromid. (3)[a] | 0 | 3 | 1 | 0.5 |
| Ammon. Iodid. (3)[a] | 3 | 3 | 1 | 0.5 |
| Ammon. Salicyl. | 0 | 5 | 1 | 0 |
| Calc. Glycerophos. | 3 | 10 | 3 | 0.5 |
| Calc. Hypophos. | 3 | 25 | 1 | 1 |
| Cerii. Oxal. | 5 | 5 | 1 | 0.5 |
| Ferr. Chlor. Soln. (3)[a] | 3 | 10 | 5 | 2 |
| Ferr. Glycerophos. | 3 | 10 | 3 | 1 |
| Ferr. Hypophos. | 5 | 24 | 3 | 1 |
| Ferr. Oxide, Rubr. | 0 | 25 | 6 | 1 |
| Ferr. Oxid. Sacch. (5)[a] | 0 | 25 | 3 | 1 |
| Ferr. Oxid. Yellow (4)[a] | 0 | 25 | 3 | 1 |
| Ferr. Phos. Sol. (3)[a] | 3 | 10 | 3 | 1 |
| Ferr. Pyrophos. Sol. (3)[a] | 0 | 10 | 3 | 1 |
| Ferr. Carb. Sacch. (5)[a] | 0 | 25 | 3 | 1 |
| Indigo Carmine | 10 | 3 | 1 | 0.5 |
| Iron (6)[a] | 0 | 25 | 3 | 1 |
| Kaolin | 0 | 3 | 1 | 0.5 |
| Lith. Benz. | 0 | 3 | 1 | 0 |
| Lith. Bromid. (1)[a] | 3 | 3 | 1 | 0.5 |
| Lith. Carb. (2)[a] | 20 | 3 | 1 | 0.5 |
| Lith. Cit. | 3 | 3 | 1 | 0.5 |
| Lith. Salicyl. | 0 | 3 | 1 | 0 |
| Mangan. Cit. Sol. | 3 | 3 | 1 | 0.5 |
| Mangan. Glycerophos. | 0 | 10 | 1 | 0.5 |
| Mangan. Hypophos. | 3 | 25 | 1 | 0.5 |
| Propylene Glycol (7)[a] | 3 | 3 | 1 | 0.5 |
| Sod. Acet. | 3 | 3 | 1 | 0.5 |
| Sod. Arsen. | 3 | 3 | 1 | 0.5 |
| Sod. Glycerophos. | 3 | 25 | 3 | 1 |
| Sod. Hypophos. | 3 | 10 | 1 | 0.5 |
| Sod. Sulf. Exsic. | 3 | 3 | 1 | 0.5 |
| Ferr. Oxid. Rub. | 0 | 3 | 1 | 0.5 |

[a] Numbers in parentheses refer to paragraphs under "Preparation of Sample."

*Procedure* (13).—To the prepared sample in a separatory funnel add the proper quantities of ammonium citrate solution, potassium cyanide solution and hydroxylamine hydrochloride solution (Table I). Add a drop of phenol red and make alkaline with ammonium hydroxide. Immediately extract the solution with 5-cc. portions of the dithizone extraction solution, draining off each extract into another separatory funnel until the lead is completely removed and the dithizone retains its original green color. Wash the combined chloroform extracts with 20 cc. of 1% nitric acid and discard the chloroform layer.

To the 1% nitric acid solution containing the lead, accurately add 4 cc. of ammonium-cyanide mixture and 5 cc. of standard dithizone solution and immediately shake for half a minute. Drain the

dithizone extract into a test tube and observe the color over a white background. This color is compared with standards which are prepared in the same manner as the substance being analyzed.

In the final estimation of lead, direct sunlight should not be allowed to strike the solution.

## DISCUSSION AND RESULTS

Some samples tested in this laboratory were also tested by the Kettering Laboratory of Applied Physiology. The Kettering Laboratory assayed the samples spectrographically, using the dithizone method, thereby giving a double check on the lead content. By and large the results of both laboratories checked very closely. Table II shows the results obtained in each laboratory.

TABLE II.—COMPARISON OF DITHIZONE AND SPECTROGRAPHIC METHODS

| Chemical | Lead Found Kettering Lab. Dithizone, P. P. M. | Lead Found Kettering Lab. Spectrograph, P. P. M. | Lead Found A. Ph. A. Lab. Dithizone, P. P. M. |
|---|---|---|---|
| Ferr. Phos. Sol. | 50 | 55 | 53 |
| Ferr. Pyrophos. Sol. | 270 | 300 | 291 |
| Ferr. Hypophos. | 0.10 | 0.10 | 0.10 |
| Peptonized Iron | 280 | 280 | 600 |

The lead content of many drugs and chemicals varies considerably from one manufacturer to another as indicated in Table III. Furthermore, there is frequently a tendency for products to vary in lead content from one batch to another in the same firm. For example, sodium arsenate was found to vary from 17 to 43 p. p. m. of lead in different lots from manufacturer B. This appears to indicate that lead control is inadequate in many instances.

TABLE III.—VARIATION IN LEAD CONTENT IN DIFFERENT SAMPLES

| Chemical | Manufacturer A Lead, P. P. M. | Manufacturer B Lead, P. P. M. | Manufacturer C[c] Lead, P. P. M. |
|---|---|---|---|
| Alum. Sulf. | 4.5 | 36 | .... |
| Ammon. Bromid. | 0.4 | 0.5 | .... |
| Ammon. Iodid. | 0.3 | 0.3 | .... |
| Ammon. Salicyl. | 0.1 | 0.1 | .... |
| Calc. Glycerophos. | .... | 0.9 | 0.6–0.7[a] |
| Calc. Hypophos. | .... | 0.1–0.25[a] | .... |
| Cerii. Oxal. | 900 | 150–225[a] | .... |
| Ferr. Chlor. Sol. | 35–80[a] | 100 | .... |
| Ferr. Glycerophos. | 250 | 13.5–60[a] | .... |
| Ferr. Hypophos. | 0.2 | 0.2 | .... |
| Ferr. Oxid. Sacch. | 100 | 1.5–14[a] | .... |
| Ferr. Phos. Sol. | 54 | 36–53[a] | .... |
| Ferr. Pyrophos. Sol. | 200–291[a] | 120 | .... |
| Ferr. Carb. Sacch. | 18 | 0.8–10[a] | .... |
| Indigo Carmine | .... | 115–834[b] | .... |
| Kaolin | 1 | 7–9 | 0.6 |
| Lith. Benz. | 0.9–1.5[a] | .... | .... |
| Lith. Bromid. | 0.5 | .... | 0.8 |
| Lith. Carb. | 0.45 | 0.5 | 0.4 |
| Lith. Salicyl. | 0.4 | .... | 0.4 |
| Mangan. Cit. Sol. | 11 | 0.6 | 2.8 |
| Mangan. Glycerophos. | 35 | 650 | 55–1850[a] |
| Mangan. Hypophos. | .... | 0.2[a] | .... |
| Sod. Acet. | 0.3 | .... | 0.3 |
| Sod. Arsen. | 5 | 17–43[a] | .... |
| Sod. Glycerophos. | 7.5 | 0.25 | 0.2 |
| Sod. Sulf. Exsic. | 0.2 | 8 | 1.5 |

[a] Two samples.
[b] Three samples.
[c] Data in this group embrace several different manufacturers and not a single firm as is the case in columns A and B.

## SUMMARY

1. A simple, rapid method for determining small amounts of lead using dithizone has been applied.

2. The test yields reliable results within reasonable limits of error and is sufficiently accurate to be used as a limit test for drug standardization.

3. Using this method, the lead content of many National Formulary drugs has been determined and recorded in this report.

4. It was shown that the lead content of many drugs varies from one lot to another and from one manufacturer to another.

5. It would appear to be advisable to consider a limit for the lead content of many of the National Formulary drugs and a level of 10 p. p. m. would appear to be a reasonable goal.

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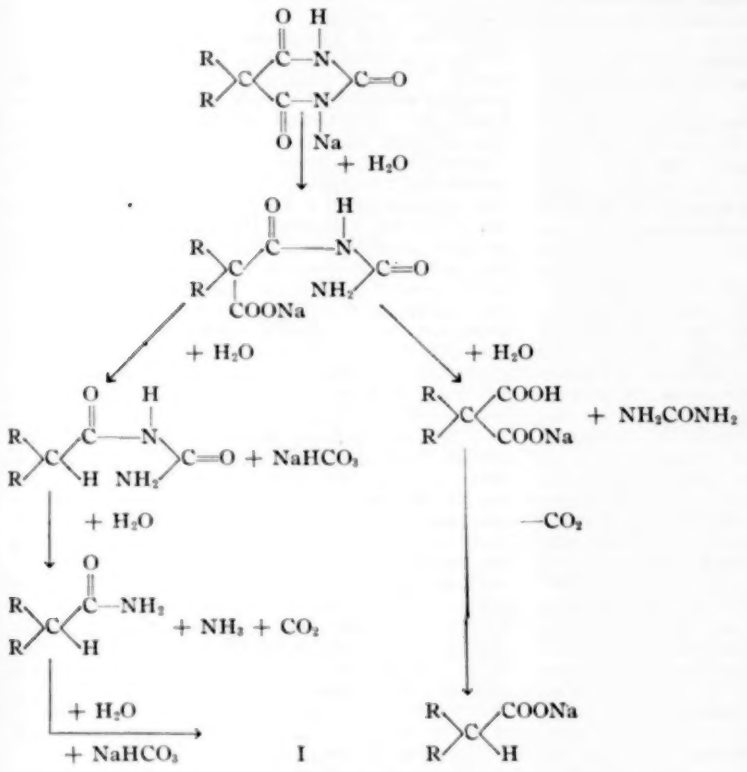
# The Stability of Solutions of Phenobarbital Sodium*,†

*By William J. Husa and Bernard B. Jatul*

One of the earliest references to the instability of malonylurea derivatives was made in 1903 by the Von Niessen Brothers (1) who found that diethylacetylurea was formed on heating diethylbarbituric acid. A complete elucidation of the decomposition



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of barbiturates in aqueous solution has been afforded by the work of Steenhauer (2), Madsen (3), Bailey (4) and Aspelund and Skoglund (5). The reactions characterizing the phenomena may be summarized from the reports of these workers as shown in Formula I.

Nielsen (6) found that the formation of the free barbituric acid in aqueous solutions of the sodium compounds takes place when the solubility of the free acid is low, as is the case with phenylethylbarbituric acid for instance.

The rate of decomposition of phenobarbital sodium has been shown by Nielsen (6), Tomski and Waller (7) and Berasain and Vitali (8) to be accelerated by increase in temperature and *p*H. According to Madsen (3) and Bailey (4) the same is true of barbital sodium.

The substances which have been reported to be stabilizing agents are diversified in nature. They include several alcohols and glycols, paraldehyde and glycerin. Various nitrogen compounds such as urethane, urea, methenamine, together with numerous amines and amides, are also mentioned in the literature.

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## EXPERIMENTAL

The present investigation was centered about the effects of time, concentration and temperature on solutions of phenobarbital sodium. The scope of the experiments represented an amplification of the

work previously reported by others. Results manifested by changes in *p*H, volume of precipitate, refractive index and percentage deterioration were studied. Undried samples were used except where otherwise indicated. The equivalent weight of dry sample upon which calculations were based was determined from the loss in weight sustained on heating other samples to constant weight at temperatures slightly over 100° C.

Before any work was carried out in this direction a method of determining the amount of undecomposed phenobarbital at any one time was sought for from the reported methods of assay. These may be classified as follows:

(*a*) Immiscible solvent methods in which the barbiturate is extracted from the aqueous media by means of a volatile solvent with or without an effort to separate the undecomposed portion from the decomposition products.

(*b*) Methods in which an undissociated compound of the barbiturate is formed by the addition of a standard solution of a heavy metal salt, the end point being evidenced in most assays by the reaction of the first excess of the latter with some reagent to produce a precipitate or a colored compound.

(*c*) Determination of the carbon dioxide and the dialkylacetylurea formed during the decomposition [Nielsen (6), Madsen (3)].

(*d*) Colorimetric determination through the agency of the formation of a colored compound with the barbiturate.

(*e*) Titration with a standard acid or alkali. Of the first class the U. S. P. XI (9) and the Rotondaro assays (10) were selected. Of the second class the methods of Budde (11), Vieböck and Fuchs (12, 13) and Schulek and Rozsa (14, 15) were studied. These methods were representative of the respective classes.

Rotondaro (10) claimed to have separated the decomposition products from the unchanged phenobarbital sodium. This was done by acidifying the solution and extracting the phenobarbital and the decomposition products with chloroform. An alkaline solution was used to remove the acidic decomposition products and the phenobarbital from the chloroform, leaving behind the phenylethylacetylurea. The phenobarbital was isolated by acidifying the alkaline solution followed by alkalization with sodium bicarbonate and extraction with chloroform, and finally removing the acidic decomposition products from the bicarbonate solution by acidifying and extraction with the same volatile solvent. The acidic decomposition products include 2,2′-phenylethylmalonuric acid, 2,2′-phenylethylmalonic acid and phenylethylacetic acid. The neutral decomposition product is phenylethylacetylurea.

A lot of phenobarbital sodium of known moisture content was assayed by the U. S. P. XI method, the Rotondaro method and a modified form of the latter. The modification consisted of chloroform extraction of the phenylethylacetylurea from an alkaline solution, rendering the residual alkaline solution acid and then alkaline with sodium bicarbonate, extraction with chloroform to isolate the phenobarbital and finally acidification of the bicarbonate solution and extraction of the acidic decomposition products. Although three U. S. P. XI assays of the lot gave an average of 90.9% recovery of phenobarbital, three Rotondaro assays gave an average of 78.6% recovery and three modified Rotondaro assays gave an average of 75.2% recovery. The maximum recovery was obtained in four subsequent modified Rotondaro assays on samples dried to constant weight at 141° C. which yielded an average of 84.7% recovery. In the U. S. P. XI method the decomposition products are extracted together with the unchanged phenobarbital. Since the possibility existed that the original sample was partially decomposed, the phenobarbital recovered in the Rotondaro assays was again subjected to the same process. The number of portions of chloroform used to extract each residue was increased from six or seven to twelve in an attempt to increase the efficiency of extraction. Each period of shaking out was not less than ten minutes. Three assays yielded an average of 75.8% recovery of phenobarbital. Three assays of Phenobarbital U. S. P. XI yielded an average of 77.0% phenobarbital by the same method. These results indicated the existence of the following possibilities:

(*a*) occurrence of decomposition during the assay,
(*b*) incomplete extraction,
(*c*) failure of the assay to separate the respective compounds quantitatively without admixture.

Assays employing silver nitrate were studied in a search for a method suitable for following the deterioration of solutions. The Budde assay (11) was conducted in the following way: About 0.2 to 0.3 Gm. of phenobarbital sodium of known moisture content was accurately weighed and dissolved in 30 cc. of water, together with 1 Gm. of reagent anhydrous sodium carbonate. While agitating vigorously with a mechanical stirrer, 0.1 *N* silver nitrate was introduced drop by drop from a microburet. The dropping was regulated so that the turbidity produced by each drop had disappeared before the introduction of the succeeding drop. The titration was carried out in a darkened room. The solution was viewed at a right angle to a strong, narrow beam of light projected through the solution. The appearance of a distinct turbidity constituted the end point. Budde (11) stated that an undissociated phenobarbital silver compound soluble in the presence of sodium carbonate was formed. In the present study it was confirmed that the silver ions are removed from the field of reaction by the formation of a weakly dissociated compound. The excess of silver nitrate is evidenced by the production of turbidity due to the reaction of excess silver ions with the sodium carbonate. Phenobarbital silver was shown to be weakly dissociated upon failure of sodium chloride to precipitate silver chloride from an alkaline solution of phenobarbital silver. It was shown that the introduction of error due to reaction of the silver nitrate with the decomposition products of phenobarbital in the concentration encountered in the solutions employed was insignificant. The Budde assay of 25-cc. portions of saturated solutions of phenylethylacetylurea yielded a distinct turbidity with 0.04 cc. of 0.1 *N* silver nitrate. A saturated solution of phenylethylacetylurea was subjected to a temperature of 127° C. for two hours at *p*H 9.9. The decomposed solution obtained thereby was titrated with 0.1 *N* silver nitrate as in the Budde method. The amount of silver nitrate consumed would have indicated 0.05% phenobarbital sodium. The same lot of phenobarbital sodium previously mentioned was assayed three times by each of the methods of Budde (11), Vieböck and Fuchs (12, 13) and Schulek and Rozsa (14, 15). The averages of the results obtained were, respectively, 89.5, 89.1 and 85.4% phenobarbital. The end point was difficult to determine in the latter two methods.

A 4% solution of Phenobarbital Sodium U. S. P. XI was heated at 115° C. for thirty minutes. The partially decomposed solution thus obtained was assayed three times by each of three methods. The U. S. P. assay showed 3.7% decomposition, the modified Rotondaro assay showed 34.7% decomposition and the Budde assay showed 16.6% de-

composition. These figures represent the averages. The U. S. P. XI method gave low results because the products of decomposition are extracted with the unchanged phenobarbital. The modified Rotondaro method gave results which were too high as shown by the failure of the method to recover all of the phenobarbital in the previous determinations. The Budde method was thereupon chosen for the determination of the percentage deterioration occurring in solutions of this barbiturate.

*Deterioration of Solutions.*—Solutions of Phenobarbital Sodium U. S. P. XI in concentrations of 5, 10 and 20% were subjected to 15-, 30-, 60- and 120-minute periods of heating at 60°, 80°, 100°, 115° and 127° C. At the end of the period the Pyrex bottles were immersed in a mixture of ice and water. A water thermostat was used for temperatures of 60° and 80° C., flowing steam at atmospheric pressure for 100° C., and an autoclave for the higher temperatures. These conditions were selected to encompass, for the most part, the official conditions of sterilization recommended for parenteral solutions by the N. F. VI (16) and the British Pharmacopœia (17). A Beckman *p*H meter, laboratory model G, was employed in all determinations of *p*H. For measurements above *p*H 9 the Beckman high *p*H, No. 1190 E, glass electrode was used. The Beckman 015 type glass electrode was used for measurements up to *p*H 9.0. A rough estimate of the volume of precipitate, which was in evidence in some of the deteriorated solutions, was made by withdrawing an aliquot portion of a vigorously agitated solution and centrifuging the aliquot in graduated centrifuge tubes at approximately 1200 r. p. m. for five minutes. The results are summarized in Table I. The percentage deterioration of each solution was calculated from an assay conducted on each of the contents of two or three bottles which were exposed as indicated in the table. The figures on *p*H and volume of precipitate represent the average of two or three determinations. The results of Tomski and Waller are summarized in Table II for the sake of comparison.

TABLE I.—EFFECT OF HEATING SOLUTIONS OF PHENOBARBITAL SODIUM U. S. P. XI[a]

| No. Min. | 60° C. | 80° C. | 100° C. | 115° C. | 127° C. |
|---|---|---|---|---|---|
| | | | 5% *w/v* Solution | | |
| 0 | 9.51 | 9.53 | 9.53 | 9.48 | 9.60 |
| 15 | 0.0 | 0.4 | 2.1 | 3.9 | 13.9 |
| | 9.51 | 9.60 | 9.43 | 9.22 | 8.81 |
| | 0 | 0 | 0 | 2 | 5 |
| 30 | 0.0 | 1.0 | 4.1 | 12.7 | 23.6 |
| | 9.49 | 9.48 | 9.23 | 8.92 | 8.65[b] |
| | 0 | 0 | 0 | 4 | 9 |
| 60 | 2.0 | 1.6 | 10.1 | 21.9 | 37.1 |
| | 9.43 | 9.37 | 9.01 | 8.68 | 8.44[b] |
| | 0 | 0 | 4 | 5 | 10 |
| 120 | .. | .. | ... | 37.0 | 56.6 |
| | .. | .. | ... | 8.52 | 8.16[b] |
| | .. | .. | ... | 10 | 17 |
| | | | 10% *w/v* Solution | | |
| 0 | .. | 9.63 | 9.62 | 9.62 | 9.75 |
| 15 | .. | 0 | 2.1 | 5.2 | 11.3 |
| | | 9.60 | 9.41 | 9.28 | 9.24 |
| | .. | 0 | 0 | 4 | 10 |
| 30 | .. | 0.4 | 5.1 | 11.1 | 22.6 |
| | .. | 9.56 | 9.23 | 8.92 | 8.73[b] |
| | .. | 0 | 4 | 10 | 18 |
| 60 | .. | 1.9 | 9.0 | 22.3 | 36.5 |
| | .. | 9.41 | 9.05 | 8.99[b] | 8.51[b] |
| | .. | 1 | 6 | 15 | 24 |
| 120 | .. | .. | ... | .. | .. |
| | | | 20% *w/v* Solution | | |
| 0 | .. | 9.71 | 9.80 | 9.76 | 9.91 |
| 15 | .. | 0.8 | 2.1 | 6.2 | 11.8 |
| | .. | 9.69 | 9.61 | 9.35 | 9.49 |
| | .. | 0 | 1 | 4 | 13 |
| 30 | .. | 1.5 | 5.3 | 11.0 | 22.3 |
| | .. | 9.65 | 9.38 | 9.12 | 9.29[b] |
| | | 0 | 5 | 11 | 17 |
| 60 | .. | 3.0 | 9.6 | 21.2 | 37.1 |
| | .. | 9.51 | 9.16 | 8.85 | 9.10[b] |
| | | 3 | 14 | 32 | 35 |
| 120 | .. | .. | ... | 35.6 | 54.0 |
| | .. | .. | ... | 8.61 | 8.36[b] |
| | .. | .. | ... | 48 | .. |

[a] The sets of three figures after each period and below a particular temperature represent in order: (*a*) per cent deterioration, (*b*) *p*H and (*c*) volume of precipitate, cc./100 cc. Blank spaces indicate that determinations were not made. After "0 min." only the *p*H of the original solution is given.

[b] Ammonia was detected.

TABLE II.—PERCENTAGE DETERIORATION FOUND BY TOMSKI AND WALLER ON HEATING 20% SOLUTIONS OF PHENOBARBITAL SODIUM CONTAINED IN AMPULS

| No. Min. | 80° C. | 100° C. | 115° C. |
|---|---|---|---|
| 15 | ... | 1.0 | ... |
| 30 | 1.0–1.5 | .... | 19.2–20.4 |
| 60 | 2.0–3.0 | 9.5–10.5 | ... |

The results in Table I indicate that the *p*H of the solution decreased on heating. The decrease was progressively greater on increase in time and temperature. In some instances the *p*H showed a slight rise which is probably explained by the presence of ammonia. Similarly the percentage deterioration and volume of precipitate increased progressively on increase in time and temperature. Keeping the temperature and time constant, the percentage deterioration generally showed little change on increase in concentration of the original solution under the conditions studied. As might be expected the refractive index underwent a decrease with each increase in amount of precipitate.

*Effect of pH.*—An experiment was carried out to learn whether or not a lowering of the *p*H would have an appreciable stabilizing influence. To a 15% solution of phenobarbital sodium various proportions of a 0.1 *M* monobasic sodium phosphate and 0.1 *M* dibasic sodium phosphate were added. For exposing the solutions to heat, 25-cc. samples were prepared in the bottles previously mentioned. The *p*H was determined before and after heating at 115° C. for thirty minutes. In some instances a precipitate was thrown out by merely lowering the *p*H. The volume of precipitate was determined through the aid of graduated centrifuge tubes as previously described.

The phosphate buffers lowered the *p*H of 5% solutions of Phenobarbital Sodium U. S. P. XI from *p*H 9.7 through various *p*H levels to *p*H 8.7. Precipitation first occurred on lowering the *p*H of unheated solutions to 8.8. The volume of precipitate produced in a 5% solution containing no buffering substances was 10 cc. per 100 cc. Although the volume of precipitate in the buffered solutions

TABLE III.—RELATION OF PHOSPHATE BUFFER TO THE PRECIPITATION IN A 5% *w/v* SOLUTION OF PHENOBARBITAL SODIUM U. S. P. XI BEFORE AND AFTER HEATING AT 115 (±1)° C. FOR 30 MINUTES

| Cc. 0.1 *M* $NaH_2PO_4$ | Cc. 0.1 *M* $Na_2HPO_4$ | Cc. 15% $C_{12}H_{11}O_3N_2Na$ | Before Heating *p*H at | Before Heating ° C. | Before Heating Ppt. Cc./100 Cc. | After Heating *p*H at | After Heating ° C. | After Heating Ppt. Cc./100 Cc.[a] |
|---|---|---|---|---|---|---|---|---|
| 0 | 40 | 20 | 9.57 | 24 | 0 | 8.70 | 25 | 8 |
| 4 | 36 | 20 | 8.78 | 25 | 0 | 8.60 | 24 | 6 |
| 8 | 32 | 20 | 8.82 | 24 | 3 | 8.62 | 25 | 4 |
| 12 | 28 | 20 | 8.80 | 25 | 6 | 8.58 | 26 | 6 |
| 16 | 24 | 20 | 8.79 | 25 | 9 | 8.63 | 26 | 6 |
| 20 | 20 | 20 | 8.80 | 25 | 9 | 8.49 | 26 | 6 |
| 24 | 16 | 20 | 8.80 | 25 | 12 | 8.54 | 26 | ..[b] |
| 28 | 12 | 20 | 8.79 | 25 | 13 | 8.51 | 25 | ..[b] |
| 32 | 8 | 20 | 8.78 | 25 | 13 | 8.57 | 25 | 6 |
| 36 | 4 | 20 | 8.76 | 25 | 14 | 8.52 | 25 | 8 |
| 40 | 0 | 20 | 8.74 | 25 | 14 | 8.52 | 25 | 6 |

[a] The *p*H of a solution of Phenobarbital Sodium U. S. P. XI prepared with the use of water instead of the buffer mixtures was 9.70 at 24° C. The volume of precipitate produced by such a solution heated under these conditions was 10 cc./100 cc.
[b] Indefinite results due to separation of a liquid and to formation of crystals during centrifuging.

which were heated was less than this figure, the reduction was not sufficient to be of practical value, as indicated in Table III.

TABLE IV.—THE STABILITY OF A 10% *w/v* SOLUTION OF PHENOBARBITAL SODIUM U. S. P. XI IN THE PRESENCE OF GLYCERIN AND ALCOHOL

| Formula | Control | *A* | *B* | *C* | *D* |
|---|---|---|---|---|---|
| Before heating | | | | | |
| % $C_{12}H_{11}O_3N_2Na$ in solution | 9.76 | 9.75 | 9.75 | 9.75 | 9.77 |
| Ppt. cc./100 cc. | 0 | 0 | 0 | 0 | 0 |
| After heating at 100 (± 0.5)° C. for 15 min. | | | | | |
| % $C_{12}H_{11}O_3N_2Na$ in solution | 9.51 | 9.62 | 9.61 | 9.61 | 9.66 |
| % deterioration | 2.6 | 1.3 | 1.4 | 1.5 | 1.1 |
| Ppt. cc./100 cc. | 1 | 0 | 0 | 1 | 1 |
| After heating at 115° C. (± 1)° C. for 30 min. | | | | | |
| % $C_{12}H_{11}O_3N_2Na$ in solution | 8.88 | 9.60 | 8.96 | 9.02 | 8.96 |
| % deterioration | 9.0 | 1.5 | 8.1 | 7.6 | 8.3 |
| Ppt. cc./100 cc. | 9 | 0 | 4 | 6 | 1 |

Solutions of Phenobarbital Sodium were prepared according to the following formulas:

*Formula A*

| | |
|---|---|
| Phenobarbital Sodium | 10 Gm. |
| Alcohol, 95% *v/v* | 50 Gm. |
| Water, distilled, *q.s. ad* | 100 cc. |

*Formula B*

| | |
|---|---|
| Phenobarbital Sodium | 10 Gm. |
| Glycerin | 50 Gm. |
| Water, distilled, *q.s. ad* | 100 cc. |

*Formula C*

| | |
|---|---|
| Phenobarbital Sodium | 10 Gm. |
| Glycerin | 20 Gm. |
| Alcohol, 95% *v/v* | 10 Gm. |
| Water, distilled, *q.s. ad* | 100 cc. |

*Formula D*

| | |
|---|---|
| Phenobarbital Sodium | 10 Gm. |
| Glycerin | 10 Gm. |
| Alcohol, 95% *v/v* | 10 Gm. |
| Water, distilled, *q.s. ad* | 100 cc. |

*Formula E*

| | |
|---|---|
| Phenobarbital Sodium | 10 Gm. |
| Glycerin | 10 Gm |
| Alcohol, 95% *v/v* | 10 Gm. |
| Antipyrine | 10 Gm. |
| Water, distilled *q.s. ad* | 100 cc. |

*Effect of Various Substances.*—Elixir of Phenobarbital U. S. P. XI could not be assayed by the Budde method. To make this possible the Tincture of Sweet Orange Peel and the Solution of Amaranth were deleted. Sufficient alcohol was added to maintain the same concentration of alcohol. Water was used to make up the difference in volume. In addition to these deletions, the syrup was omitted in another formula. Four 35-cc. portions of each of the above solutions were placed in four-ounce glass-stoppered Pyrex bottles. Two bottles of each solution were heated for each of two periods. The conditions were fifteen minutes at 100° C. and thirty minutes at 115° C. No deterioration could be detected by the Budde assay in those elixirs in which the above-mentioned deletions had been made. No precipitate was produced in the official elixir nor in an elixir containing 0.4% methenamine but without the Tincture of Sweet Orange Peel and the Solution of Amaranth.

The results produced on heating this solution are summarized in Table IV. Each figure given in this table represents the average of two determinations.

The solution containing 46% *w/v* alcohol showed 1.5% deterioration after heating at 115° C. for thirty minutes whereas the other solutions showed approximately 8% deterioration which was only slightly less than that shown by the control solution of 10% Phenobarbital Sodium U. S. P. XI in water alone. On heating at 100° C. for fifteen minutes somewhat less deterioration occurred in all of the formulas than in the control. The solution containing antipyrine could not be assayed by the Budde method. No precipitate was evident after heating at 100° C. for fifteen minutes; there was 1 cc. of precipitate per 100 cc. after heating at 115° C. for thirty minutes.

Ten per cent solutions of Phenobarbital Sodium U. S. P. XI containing 50% Dextrose U. S. P. XI were prepared. One was made with water, another

with 9% *w/v* alcohol and the last with 10% *w/v* glycerin. In the bottles previously mentioned 35-cc. portions were heated at 100° C. for fifteen minutes. Duplicates were run. The percentage deterioration in the control was 2.4% whereas the dextrose solutions showed, respectively, 9.0, 1.8 and 7.1% deterioration. The dextrose solutions had turned brown. The solutions were considered unsatisfactory.

Ten per cent solutions of Phenobarbital Sodium U. S. P. XI containing 40, 55 and 70% *v/v* propylene glycol were prepared. The solutions were heated at 100° C. for fifteen minutes. Duplicates were run. The control showed 2.0% deterioration whereas the propylene glycol solutions showed 0.1, 0.0 and 0.6% deterioration, respectively. A light amber hue was evident in all of the solutions containing the glycol. Berasain and Vitali (8) had claimed that a 10% solution of this barbiturate containing 40% *v/v* propylene glycol was stable as evidenced by the absence of a precipitate on heating at 100° C. for fifteen minutes and on storage for six months.

## DISCUSSION OF RESULTS

*Effect of Heat.*—The use of heat to sterilize solutions of phenobarbital sodium causes deterioration, the extent of which is a function of temperature and time.

*Effect of pH.*—Solutions of phenobarbital sodium are markedly alkaline, the *p*H values of 5, 10 and 20 per cent solutions being 9.5, 9.7 and 9.8, respectively. No data were found in the literature as to how much the *p*H could be lowered before precipitation of phenobarbital would commence. Our results indicate that on addition of a buffer solution containing monosodium and disodium phosphates, a precipitate first appears at *p*H 8.8. Previous authors (6, 8) have reported a decrease in rate of hydrolysis and quantity of precipitate in solutions of lowered *p*H. Our results show that there is a slight decrease in precipitation on heating solutions of lowered *p*H. However, the stabilizing effect is too slight to be of practical value.

*Effect of Stabilizers.*—Hydroalcoholic solutions of phenobarbital sodium are more stable toward heat than aqueous solutions. After heating at 115° C. for thirty minutes, a solution containing 46 *w/v* per cent of alcohol shows 1.5 per cent deterioration as compared with 9.0 per cent deterioration in an aqueous solution.

The addition of dextrose to the extent of 50 per cent to solutions of phenobarbital sodium does not assure stability. Moreover, the solutions are considered unsatisfactory because of the brown color which develops on heating.

Solutions of phenobarbital sodium containing a high proportion of propylene glycol are considerably more stable than aqueous solutions. These results are in accord with the experiments of Berasain and Vitali (8). However, our results show that the solutions containing the glycol may acquire a light amber color upon heating.

## SUMMARY

A study was made of the deterioration of solutions of phenobarbital sodium. The effect of various factors such as time, temperature, concentration, *p*H and presence of stabilizers was determined. The decomposition was followed by chemical assay as well as by noting the volume of precipitate and the changes in refractive index and *p*H.

Heat causes deterioration, the extent of decomposition depending on the temperature and time of heating. A lowering of *p*H decreases the rate of deterioration but not sufficiently to be of practical value. At *p*H values of 8.8 and less, there is precipitation of phenobarbital.

By comparative tests of various stabilizers, it was found that hydroalcoholic solutions are more stable toward heat than aqueous solutions.

Solutions of phenobarbital sodium containing a high proportion of propylene glycol are more stable than aqueous solutions.

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# The Effect of Age upon the *p*H of Dilute Solutions of Zinc Sulfate and Solutions of Mild Silver Protein*

*By Charles V. Netz*

Physicians use very dilute solutions of zinc sulfate, alone and with other agents, as ophthalmic astringents. A 0.22 per cent solution of zinc sulfate appears to be the most popular. The astringency of zinc sulfate solutions is due to the zinc ion. Astringent substances will irritate the delicate corneal membrane; hence dilute solutions of zinc sulfate are somewhat irritating to the eye, and the irritation is proportional to the concentration of the zinc ion. A number of local physicians have reported that solutions of zinc sulfate, as they become older in office use, appear to cause increased corneal irritation. Since zinc sulfate is completely ionized in solution, the concentration of the zinc ions should not increase with time, hence there should be no increase in the astringency of such a solution with age, providing there is no evaporation of the solvent.

Dilute solutions of zinc sulfate are weakly acid due to the slight hydrolysis of the zinc ions which causes an excess of hydronium ions. Although hydronium ions are irritating to the cornea they are reasonably well tolerated because a half-saturated solution of boric acid with a *p*H of 4.8 is only mildly irritating. Observations were made over a period of fifteen months on three 0.22 per cent solutions of zinc sulfate. A 1500-cc. portion of the solution was prepared and from this were filled three pint bottles of ordinary clear glass. Bottles of this type were used to simulate the conditions under which such solutions are usually found in physicians' offices. The bottles were kept in daylight but not in direct sunlight. The *p*H values of the solutions were determined at intervals with a Coleman Electrometer and glass electrode. A very slight precipitate which slowly formed was not filtered out. The temperatures of the solutions ranged from 21° to 23° C.

The distilled water used to make the solution had a *p*H of 6.74. The freshly prepared 0.22 per cent solution of zinc sulfate had a *p*H of 6.70. The *p*H values of the solutions uniformly decreased (solution became more acid) until at the end of the fifteen months of observation each had a *p*H of 6.38. Obviously the slight increase in acidity over that period could hardly be the cause of additional corneal irritation. Five people who dropped in their eyes on alternate days a freshly prepared 0.22 per cent solution of the salt and the fifteen-month-old solution could discern no difference in the astringency of the solutions.

The increased irritation of old solutions of zinc sulfate as reported by a few physicians is certainly not due to increased acidity. It may be due to an increase in zinc ions caused by loss of solvent from containers which are poorly stoppered or are opened for brief periods during routine office use.

The *p*H of a 5 per cent solution of mild silver protein was determined at intervals over a period of twelve months, using the same general procedure as outlined above for zinc sulfate solutions. The *p*H of the solution in each bottle decreased (became less alkaline) from 9.65 at the time of preparation to 9.05 after twelve months of standing.

## SUMMARY

1. The *p*H of three 0.22 per cent solutions of zinc sulfate decreased from 6.70 to 6.38 over a fifteen-month period.

2. The *p*H of two 5 per cent solutions of mild silver protein decreased from 9.65 to 9.05 over a twelve-month period.

* Received July 30, 1943, from the College of Pharmacy, University of Minnesota, Minneapolis.

# A New Method for the Preparation of Pyridine-2,5-Dicarboxylic Acid*

*By Taito O. Soine*

During studies on the oxidation of quinaldine (2-methyl quinoline) it was found that a drastic oxidation using concentrated sulfuric acid and concentrated nitric acid resulted in the formation of good yields of pyridine-2,5-dicarboxylic acid. Previous methods of preparation have involved the oxidation of 2-methyl-5-ethyl pyridine (1, 2, 3), 2,5-dimethyl pyridine (4, 5, 6, 7), 2,3,6-pyridine tricarboxylic acid (8) or crude lutidine (9), all of which are somewhat difficult to obtain. Quinaldine is easily prepared by the Doebner-von Miller synthesis or is available commercially.

## EXPERIMENTAL

*Quinaldine.*—This was prepared by the Doebner-von Miller synthesis (10) using crotonaldehyde as one of the reactants instead of acetaldehyde or paraldehyde. A 50% yield of a product, b. p. 243–245°, was obtained.

*Pyridine-2,5-dicarboxylic Acid.*—Twenty cubic centimeters of quinaldine was very slowly poured into 40 cc. of concentrated sulfuric acid in a 500-cc. Erlenmeyer flask. The mixture was cooled and concentrated nitric acid added in 1- to 2-cc. portions until addition of the acid caused no further evolution of brown fumes. The mixture was then warmed on a hot plate at low heat until brown fumes were evolved, and when the evolution of the fumes subsided more nitric acid was added in 1- to 2-cc. portions. By gradually increasing the heat during the additions of nitric acid, a temperature of 230–240° was reached, at which point care was needed to prevent the mixture from carbonizing excessively. It was desirable, however, to produce a small amount of carbonization, and then to discharge it by cooling to 190–200° and adding more nitric acid in small amounts. Finally, the mixture became yellow and remained that way at 230–240°. At this point the mixture was continuously oxidized by the addition of 1- to 2-cc. portions of nitric acid for three hours or until the evolution of brown fumes was quite negligible.

The whole oxidation procedure required seven to eight hours and at least one-half to two-thirds of the oxidation took place at 230–240°. The total amount of nitric acid required was approximately 300 cc. The oxidized mixture was then cooled to 25° and about two volumes of distilled water added. By allowing this highly acid mixture to stand for a short period of time it was possible to obtain a small yield of crystalline pyridine-2,5-dicarboxylic acid directly. However, it was found to be faster and productive of better yields to reduce the acidity by means of alkali to a point where a maximum amount of the crude acid was precipitated. Therefore, fifty per cent sodium hydroxide was added to the mixture (splattering) until precipitation was complete, and then the mixture was cooled to room temperature.

The amount of alkali added should be such that the mixture remains distinctly acid. Usually a small amount of some substance (nitroquinaldinic acid?) is present, and serves as an indicator because it is red in alkaline solution. The precipitate was filtered off, washed several times with cold distilled water and dried. The yield was 14.5 Gm. of crude acid (62% of the theoretical) and was tinged with a light yellow color. The crude acid was dissolved in 900 cc. of distilled water, and boiled with decolorizing carbon (Norit) for fifteen minutes. On filtering and cooling the filtrate, 13.4 Gm. of small, white crystals, m. p. 238°, was deposited. The literature reports two different melting points, 236° and 254°, the apparent discrepancy being due to the presence of water of crystallization in the lower melting one (5).

*Pyridine-2,5-dicarboxylic Acid Dichloride.*—Two grams of the recrystallized acid was treated with 20 cc. of thionyl chloride and refluxed on a steam bath for ten hours or until a solution had formed. The excess thionyl chloride was then distilled off on a steam bath and the residue evacuated in a desiccator. Upon standing for a day or two under vacuum the residue crystallized into long needles. These were not purified further, but were used to prepare the dimethyl ester, and the diamide for purposes of identification.

*Dimethyl Ester of Pyridine-2,5-dicarboxylic Acid.*—One-half of the crystalline dichloride was treated with an excess of anhydrous methyl alcohol. The dimethyl ester was precipitated from the methyl alcohol solution by addition of an excess of water. The crystalline precipitate was recrystallized from methyl alcohol to yield colorless needles, m. p. 161–163°. The literature (11) reports 162–163°.

*Diamide of Pyridine-2,5-dicarboxylic Acid.*—The remainder of the crystalline dichloride was treated with an excess of 28% ammonia, and the insoluble precipitate filtered off and dried. It was recrystallized from nitrobenzene to yield small needles, m. p. 310–313° (with decompn.). The literature (12) reports 310° (with decompn.).

## SUMMARY

1. A new method of preparing pyridine-2,5-dicarboxylic acid by the oxidation of quinaldine with concentrated sulfuric and concentrated nitric acids is described.

2. The dimethyl ester of the acid and the diamide of the acid were prepared to support the identification of the acid.

* Received Jan. 9, 1944, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minn.

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# Book Review

*Virus Diseases in Man, Animal and Plant*, by GUSTAV SEIFFERT. Philosophical Library Inc., New York, N. Y., 1944. 332 pp., 14.5 x 23 cm. Price, $5.00.

This book is an English translation by Dr. Marion Lee Taylor and was published upon recommendation of the National Research Council. It is an attempt to review and correlate the more important investigation of the past decade in the field of virus diseases. The virus problem is still in the stage of development and much of the available information is uncertain and unclarified. In view of this fact the author has presented a remarkably arranged survey which should serve as an excellent guide to those who wish to become more closely acquainted with the virus problem.

In a general division of 90 pages the author discusses the conception of "virus" and historical data and includes discussions with references to the literature covering manifestations and nature of virus, elementary corpuscles, inclusion corpuscles, measurement of size and filterability of virus, production of virus in chemically pure crystalline form, concentration and purification of virus, behavior of vira toward chemical and physical influences, variability, pathogenesis of virus diseases, natural and acquired immunity, protective inoculation and epidemiology of virus diseases.

Following these general discussions is a special division devoted to specific diseases of man, mammals, birds, insects and plants. Each disease is discussed in an individual chapter and conclusions are drawn from the literature quoted. In many cases the unsolved problems are carefully noted. Virus-like organisms and filterable types of bacteria are discussed in special divisions.

In order to stimulate individual investigation on the part of the reader, the author has included a section on methods of virus investigation. General facts of virus investigation are discussed and detailed descriptions are presented of methods for identifying elementary and inclusion corpuscles, methods of filtration, the production and use of gradocoll filters, methods of virus cultivation, and important serological methods such as proof of virus-neutralizing substances in the serum, agglutination, precipitation, complement binding and cross immunization testing.

All in all the author has presented a well-balanced survey of the present state of virus research. The book is decidedly readable and should serve not only as an informative review, but also as a stimulus for the worker to attack the many as yet unsolved problems in this field. Seven figures are included and an excellent reference index concludes the book.
—ELMER H. WIRTH.

## WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Arachidonic acid
*p*-Cyclohexyl acetanilide
*p*-Phenetyl urea (Dulcin)
3,4-Dihydroxydiphenyl amine
7,8-Dihydroxy-1,4-naphthoquinone
$\beta,\beta'$-Dipyridyl
Folic acid
1,2,4,5,6,8-Hexahydroxy anthraquinone
Mesoxalic acid
Mellitic acid (100 Gm.)
9-Methyl-2,6,7-trihydroxy-3-fluorone
Protalbinic acid
Purpuric acid
Quinaldic acid
Sulfapyrazine
Sodium quinizarin-2,6-disulfonate
Tri-iodo acetic acid (100 Gm.)
1,2,5,8-Tetrahydroxy anthraquinone